

(15)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) Publication number:

0 466 251 A1

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: **91201702.7**

(51) Int. Cl.⁵: **C12N 15/31, C12P 21/02,
C12N 1/21, C12R 1/465,
C12P 19/02**

(22) Date of filing: **02.07.91**

(30) Priority: **10.07.90 EP 90201851**

(43) Date of publication of application:
15.01.92 Bulletin 92/03

(94) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(71) Applicant: **GIST-BROCADES N.V.
Wateringseweg 1 P.O. Box 1
NL-2600 MA Delft(NL)**

(72) Inventor: **Luiten, Rudol Gijsbertus Marie
Akkerhoornbloem 26
NL-2317 KR Leiden(NL)
Inventor: Kerkman, Richard
Scheepersstraat 60
NL-2021 BK Haarlem(NL)
Inventor: Bosch, Leendert
Hofbrouckerlaan 56
NL-2341 LR Oegstgeest(NL)
Inventor: Vijgenboom, Erik
Rozenoord 62
NL-2651 XP Berkel en Rodenrijs(NL)
Inventor: Heinstra, Pieter Wilhelmus
Hendrikus
Eykmanlaan 241
NL-3571 JH Utrecht(NL)
Inventor: Woudt, Lambertus P.
Schouw 2
NL-1613 CM Grootebroek(NL)**

(74) Representative: **Huygens, Arthur Victor, Dr. et
al
c/o Gist-Brocades N.V. Patent & Trademarks
Department Wateringseweg 1 PO Box 1
NL-2600 MA Delft(NL)**

(54) **Elfamycin-resistant mutants.**

(57) Elfamycin-producing actinomycetes, in particular the mocimycin-producing streptomycetes, are frequently too sensitive for the elfamycin produced by them. This limiting factor for the production of the elfamycin concerned is removed by mutating the gene *tuf*, encoding the protein EF-Tu, into a gene *tufR*, encoding a protein EF-TuR, which protein is resistant to the elfamycin concerned. The gene *tufR* is expressed in host cells which then show an increased resistance to the elfamycins tested.

EP 0 466 251 A1

The present invention relates to elfamycin producing actinomycetes, to a protein EF-Tu (Elongation Factor Tu) of an elfamycin producing actinomycete, to the DNA sequence *tuf* encoding this protein, to replicable vectors containing this DNA sequence and to actinomycetes transformed with these vectors.

The elfamycins are a group of antibiotics, to which belong Mocimycin (also known as Kirromycin), Dihydromocimycin, N-Methylmocimycin (also known as Aurodox), Kirrothricin, Azdimycin, Efrotomycin, and Pulvomycin. They are produced by bacteria belonging to the order of the Actinomycetales. In particular, the elfamycin antibiotic mocimycin, subject matter of British Patent 1325200, is produced by bacteria belonging to the genus *Streptomyces*, such as *Streptomyces collinus*, *Streptomyces diastatochromogenes*, *Streptomyces fradiae* and especially *Streptomyces ramocissimus*.

In practice, the level of production of elfamycins, in particular mocimycin, by the above bacteria is often found to be too low, so as to make their commercial exploitation unattractive.

The antibiotic action of the elfamycins, including mocimycin, is known to be due to their inhibition of EF-Tu (H. Wolf et al., Proc. Natl. Acad. Sci. USA, 75 (1978) 5324-5328).

The Polypeptide Chain Elongation Factors (EF) are essential for cellular protein synthesis. The type designated EF-Tu occurs in all prokaryotic cells, including gram-negative bacteria such as *Escherichia coli* and gram-positive bacteria such as those belonging to the order of Actinomycetales. Different organisms have similar, but not identical, EF-Tu. The DNA sequence encoding EF-Tu has been designated the *tuf* gene.

It was further found by C. Glöckner and H. Wolf (FEMS Microbiology Letters 25 (1984) 121-124), that the EF-Tu isolated from all tested mocimycin producing strains of the genus *Streptomyces* was sensitive to relatively low concentrations of elfamycin in a cell-free protein synthesizing system. On the other hand, these authors found the EF-Tu isolated from the kirrothricin producing *Streptomyces cinnamomeus* and from the efrotomycin-producing *Streptomyces lactamdurans* (recently renamed *Nocardia lactamdurans*) to be relatively resistant not only to the endogenous antibiotic but also to mocimycin. These authors suggested that the sensitivity of the EF-Tu of the elfamycin producing strains to their own elfamycin is the limiting factor of their production capacity. They speculated that strains such as *Streptomyces cinnamomeus* and *Streptomyces lactamdurans* may be suitable sources of mutants with increased productivity because they tolerate high antibiotic levels in the cell.

Mutagenesis, by chemical mutagenic compounds, of originally elfamycin sensitive EF-Tu into EF-Tu exhibiting an increased resistance to elfamycin, was described by E. Fischer et al. (Proc. Natl. Acad. Sci. USA, 74 (1977) 4341-4345) in a laboratory strain of *E. coli* having altered membrane permeability, and also by J.A.M. van de Klundert et al. (FEBS Letters 81 (1977) 303-307). The first-mentioned authors report the mutant *E. coli* to be deficient in growth capacity (in the absence of elfamycin), when compared to the parent *E. coli*. The mutation leading to the increased elfamycin resistance of *E. coli* EF-Tu was found to be a change of an alanine residue at position 375 to either valine or threonine (F.J. Duisterwinkel et al., EMBO J. 3 (1984) 113-120). No other elfamycin resistant EF-Tu proteins have been reported from other gram-negative bacteria; an elfamycin resistant EF-Tu from the gram-positive bacterium *Bacillus subtilis* has been identified, but not characterized at the molecular level (I. Smith and P. Paress, J. Bacteriol. 135 (1978) 1107-1117). No such mutations have been described in any actinomycete, especially in streptomycetes, in particular in mocimycin producing streptomycetes, more in particular in *Streptomyces ramocissimus*.

In the two above-mentioned publications about chemical mutagenesis of *E. coli* strains leading to an increased resistance to elfamycin, it is disclosed that *E. coli* has two closely related but distinct EF-Tu proteins, originating from two distinct *tuf* genes. Since elfamycin inhibits the activity of sensitive EF-Tu by binding it irreversibly to the ribosomes, it follows on theoretical grounds that an elfamycin resistant mutant of *E. coli* has to have either the two EF-Tu proteins both mutated and active, or only one of them mutated and active, the other one then being non-active. This was confirmed by *in vitro* experiments.

In streptomycetes, in particular in *Streptomyces ramocissimus* the present inventors have identified three closely related *tuf*-genes. On further investigation it was discovered that one of those is mainly expressed in the vegetative mycelium of the streptomycete. The protein products of both other genes constitute approximately 5 percent of the amount of the main EF-Tu species. This has specifically been observed in *Streptomyces ramocissimus*. A similar protein pattern was found in *Streptomyces collinus*, and *Streptomyces goldiniensis*.

In contrast to *Escherichia coli*, streptomycetes have the capacity to undergo a complex morphological and biochemical differentiation towards spore formation. It is therefore conceivable that during the sporulation and subsequent germination process (one of) the minor vegetative EF-Tu species becomes the main active EF-Tu. This differential expression would then be analogous to e.g. the expression of different sigma factors observed in *B. subtilis* (R. Losick et al., Ann. Rev. Genetics 20 (1986) 625-669) and *S. coelicolor* A3-(2) (M.J. Buttner, Molecular Microbiol. 3 (1989) 1653-1659) directing the transcription of developmentally

regulated sets of genes. Differential expression of EF-Tu encoding genes may be required to adapt the translation machinery to specific requirements of the developmental phase.

Even though the level of expression of the two minor EF-Tu species in the vegetative mycelium is low, it is still possible that, in analogy to the situation in *Escherichia coli*, they convey the dominance of the elfamycin sensitivity even if the major EF-Tu protein is rendered elfamycin resistant; the relative level of elfamycin sensitive EF-Tu versus elfamycin resistant EF-Tu at which an elfamycin resistance phenotype becomes apparent is unknown. For the purpose of the present invention however, streptomycetes, in particular *Streptomyces ramocissimus* is considered to have one major EF-Tu.

It has now been found possible to modify the elfamycin sensitive EF-Tu protein of an elfamycin producing actinomycete, in particular a mocimycin producing streptomycete, more in particular one belonging to the species *Streptomyces ramocissimus*, thereby conferring to this protein an increased resistance to the elfamycin produced by this bacterium. This has been found possible to achieve by mutagenesis. In particular, the present inventors have achieved the mutagenesis by using site-directed mutagenesis techniques for modifying the original gene *tuf*, encoding the elfamycin sensitive EF-Tu, to a novel gene *tufR*, encoding a novel protein EF-TuR having an increased resistance to the elfamycin.

The present invention therefore provides proteins EF-TuR, characterized in that they have been derived from an elfamycin producing actinomycete and made resistant to the elfamycin by mutagenesis, in particular site-directed mutagenesis.

The invention further provides various DNA sequences *tufR*, encoding said proteins EF-TuR.

The invention still further provides vectors, containing said DNA sequences. Such vectors are replicable and/or capable of integrating into the chromosomal DNA sequence of an elfamycin producing actinomycete.

The invention yet further provides an elfamycin producing actinomycete, comprising a DNA sequence *tufR* instead of the DNA sequence *tuf*. Such actinomycetes have been found to possess a substantially increased resistance to the elfamycin. The increased resistance of the EF-Tu protein to elfamycin removes a limiting factor in the elfamycin production. The expression of the elfamycin resistant *tuf* genes is found to influence the growth of the transformed strains.

The invention further provides processes for the preparation of said DNA sequences, vectors, and actinomycete.

30 Description of the drawings

In the drawings the following abbreviations and symbols are used:

- bla** : ampicillin resistance gene (**bla**- if inactive);
- bla*** : position of translation stopcodon in **bla**-;
- 35 **cat** : chloramphenicol resistance gene (**cat**- if inactive);
- cat*** : position of translation stopcodon in **cat**-;
- tsr** : thiostrepton resistance gene;
- ori322** : replication origin derived from plasmid pBR322;
- orif1** : replication origin derived from phage f1;
- 40 **rep660** : replication origin region derived from plasmid pMT660;
- Srtuf** : *S. ramocissimus* *tuf* gene, **Srtuf 3'** if only the 3' coding region of the gene is present;
- Plac** : *E. coli* *lac* operon promoter.

Figure 1

45 The DNA sequence of the *S. ramocissimus* *tuf1* gene and the amino acid sequence of the *S. ramocissimus* EF-Tu1 protein derived therefrom (also shown as SEQ ID NO : 1).

EF-TuR is characterized here by replacement of the amino acid alanine at position 378 by valine or threonine, respectively.

50 The gene *tufR* is characterized here in that the codon encoding alanine at position 378 (GCC) is changed to codons encoding valine, threonine, proline, or phenylalanine.

Figure 2

- 55 a. Map of plasmid pUSrT1.
- b. Map of expression plasmid pUSrT1-1. In plasmids pUSrT1V-1, pUSrT1T-1, pUSrT1P-1, and pUSrT1F-1, Ala378 is replaced by valine, threonine, proline, or phenylalanine, respectively.

Figure 3

- a. Map of plasmid pMaSrT1. In plasmids pMaSrT1V, pMaSrT1T, pMaSrT1P, and pMaSrT1F, Ala378 is replaced by valine, threonine, proline, and phenylalanine, respectively.
- b. Map of plasmid pMcSrT1.

Figure 4

- a. Graphic representation of residual activities of SrEF-Tu and SrEF-Tu mutants A378V and A378T in an *in vitro* polyphenylalanine synthesizing system, in the presence of different concentrations of mocimycin.
- b. Graphic representation of the time course of incorporation of ³H-phenylalanine in an *in vitro* polyphenylalanine synthesizing system by SrEF-Tu and SrEF-Tu mutants A378V and A378T in the presence of 16 mg/l mocimycin.

Figure 5

- a. Analysis of the mutant SrEF-Tu1 proteins A378V and A378T in the elfamycin binding assay. Lanes 1 and 2: wild-type SrEF-Tu, lanes 3 and 4: SrEF-Tu A378T, lanes 5 and 6: SrEF-Tu A378V. In lanes 2, 4, and 6 the indicated SrEF-Tu protein have been pre-incubated with 25 μ M aurodox.
- b. Analysis of the mutant SrEF-Tu1 proteins A378P and A378F in the elfamycin binding assay. Lanes 1 and 2: wild-type SrEF-Tu, lanes 3 and 4: SrEF-Tu A378F, lanes 5 and 6: SrEF-Tu A378P. In lanes 2, 4, and 6 the indicated SrEF-Tu protein have been pre-incubated with 25 μ M Aurodox.

Figure 6

- a. Map of plasmid pUt18.
- b. Map of plasmid pStT1-1. In plasmids pStT1V-1 and pStT1T-1, Ala378 is replaced by valine and threonine, respectively.

Figure 7

- a. Map of plasmid pStT1 Δ S. In plasmids pStT1V Δ S and pStT1T Δ S, Ala378 is replaced by valine and threonine, respectively.
- b. Map of plasmid pMTST1 Δ S. In plasmids pMTST1V Δ S and pMTST1T Δ S, Ala378 is replaced by valine and threonine, respectively.

Figure 8

- a. Map of the *S. ramocissimus* CBS 190.69 chromosomal *tuf* locus.
- b. Map of the *S. ramocissimus* *tuf* locus in which plasmid pMTST1V Δ S is integrated via homologous recombination.
- c. Map of the *S. ramocissimus* R1V chromosomal *tufR* locus.

Figure 9

Analysis of the SrEF-Tu from *S. ramocissimus* strain R1V with respect to elfamycin binding. Samples were loaded on the native gel as follows: Lanes 1 and 2: wild-type SrEF-Tu isolated from *E. coli* JM101-[pUSrT1-1], lanes 3 and 4: SrEF-Tu isolated from *S. ramocissimus* CBS 190.69, lanes 5 and 6: SrEF-Tu isolated from *S. ramocissimus* strain R1V. In lanes 1, 4, and 6, the indicated SrEF-Tu proteins were pre-incubated with 25 μ M Aurodox.

Detailed description of the invention

Elfamycin producing species can be found among the Actinomycetes. Preferably Streptomycetes are used. Examples are the mocimycin producing streptomycetes *Streptomyces collinus*, *Streptomyces diastatochromogenes*, *Streptomyces fradiae*, and *Streptomyces ramocissimus*. Most preferably *S. ramocissimus* is used.

Elongation factor Tu (EF-Tu) can be isolated in a number of ways. For example different combinations

of general protein purification techniques known in the art, such as stepwise ammonium sulphate precipitation, gel filtration, and ion-exchange chromatography can be used. Application of this approach for the purification of EF-Tu protein have been described by D. Miller and H. Weissbach (Arch. Biochem. Biophys. 141 (1970) 26-37), K.-I. Arai et al. (J. Biol. Chem. 247 (1972) 7029-7037), and R. Leberman et al. (Anal. Biochem. 104 (1980) 29-36). A preferred isolation procedure for EF-Tu is the following. After culturing *S. ramocissimus* the mycelium is harvested by centrifugation. The mycelium is resuspended and sonicated. After differential centrifugation to remove the ribosomes, the protein is further purified by affinity chromatography (G. Jacobson and J. Rosenbusch, FEBS Lett. 79 (1977) 8-10); for this purpose GDP-AH-Sepharose is especially useful. After purification the protein is further characterized by GDP exchange analysis (H. Weissbach et al., Arch. Biochem. Biophys. 137 (1970) 262-269) and by its ability to promote EF-Tu dependent peptide synthesis in a cell-free extract, e.g. as described by C. Glöckner and H. Wolf (cited above). Further characterization can be performed by determination of the amino acid composition and (partial) amino acid sequence of the protein.

Susceptibility of the isolated EF-Tu to elfamycin is tested in elfamycin binding studies (G. Chinali et al., Eur. J. Biochem. 75 (1977) 55-65,) and in studies on the inhibition of EF-Tu dependent peptide synthesis (C. Glöckner and H. Wolf, cited above). Still another direct elfamycin binding assay has been developed in which the capacity of the EF-Tu protein to bind elfamycins can be visualized by a change in EF-Tu protein migration in the presence of the elfamycin by non-denaturing PAGE (polyacrylamide gel electrophoresis). Upon elfamycin binding to EF-Tu.GDP, a ternary complex is formed which is more negatively charged than the binary EF-Tu.GDP complex (B. Kraal et al., 1989, in The Guanine-Nucleotide Binding Proteins, pp 121-129, Plenum Press, New York). Consequently elfamycin binding, using e.g. aurodox, to *Escherichia coli* wild-type EF-Tu increases the migration distance into a non-denaturing polyacrylamide gel. Both the methods of peptide synthesis inhibition and visualization of elfamycin binding are the preferred techniques to determine the effect of elfamycin binding on EF-Tu functioning. These susceptibility assays are important if increased elfamycin resistance of EF-Tu has to be established.

Several ways are possible to obtain mutants of EF-Tu exhibiting an increased resistance to elfamycin. One of them is mutagenesis of the parent microorganism. This can be performed by for example chemicals, such as ethyl methane sulphonate and N-methyl-N'-nitro-N-nitrosoguanidine, or by UV irradiation. Subsequent selection for increased elfamycin resistance can yield strains that contain EF-Tu with an increased resistance to elfamycin. This can be tested by isolating the protein and performing on it elfamycin binding studies, and by EF-Tu dependent peptide synthesis as described above. Another way of performing the mutagenesis is on the cloned gene coding for the EF-Tu. In this approach it is possible to randomly mutagenize this gene by chemical (R. Myers et al., Science 229 (1985) 242-247) or enzymatic means (P. Lehtovaara et al., Protein Engineering 2 (1988) 63-68), or to focus mutagenesis on one or more specific regions/nucleotides of the gene (site-directed mutagenesis). Site-directed mutagenesis is the preferred embodiment of the present invention.

For cloning the gene encoding the EF-Tu, chromosomal DNA from the relevant elfamycin producing species is isolated and inserted in a suitable vector. Possible vectors are among others plasmids, phages, and cosmids. If necessary, expression vectors can be used. The clones containing the *tuf* genes can be selected via hybridization with synthetic probes, which are synthesized according to previously determined protein or partial protein sequences. It is also possible to use *tuf* genes isolated from other species as hybridization probes, provided that there is sufficient similarity between the two genes. It is assumed that when 80% identity exists at the protein level there will be enough identity at the DNA level to detect homologous genes by hybridization. Hence genes from other species that can be found by hybridization and that encode a protein having elongation factor activity are also covered by the present invention. Upon cloning in an expression vector it also becomes possible to screen the DNA library thus obtained using antibodies specific to EF-Tu. Preferably the chromosomal DNA from *S. ramocissimus* is isolated (as described by D. Hopwood et al. (1985) Genetic Manipulation of Streptomyces: A Laboratory Manual, John Innes Foundation, Norwich) and cloned in a plasmid such as pUC8 or pUC18. Selection of one *tuf* gene is performed using the HpaI/NruI fragment of the *E. coli* *tufA* gene as a hybridization probe (T. Yokota et al., Gene 33 (1980) 25-31). At a later stage the first *S. ramocissimus* *tuf* gene was used as a probe. In this way three *tuf* genes were detected, cloned, and then sequenced using the Sanger method (Proc. Natl. Acad. Sci. USA 74 (1977) 5463-5467). By using specific probes derived from these three sequences in a Northern blotting experiment, transcription of only one of the *tuf* genes was detected during vegetative growth of *S. ramocissimus*. This main functional *S. ramocissimus* *tuf* gene (*Srtuf1*) was found to be located on a 2.8 kb BglII chromosomal restriction fragment. Furthermore, using specific antibodies, it was found that the protein encoded by the *Srtuf1* gene was approximately 20 times more abundant than the proteins encoded by the other two genes. The latter genes, called *Srtuf2* and *Srtuf3*, were encoded on a 3.0 kb BamHI fragment and

a 4.2 kb PstI fragment, respectively.

In order to test whether the elfamycin resistance of the SrEF-Tu1 could be improved, site-directed mutagenesis was applied on the Srtuf gene. From comparison of EF-Tu sequences found in different species it is possible to make a prediction which amino acids are important. Other ways to achieve this may be the analysis of the three-dimensional structure of the protein, inhibitor studies or enzymatic mechanism studies. From the information thus obtained specific mutations can be proposed.

For the elfamycin resistant *E. coli* strains mentioned above it has been found that replacement of the amino acid alanine at position 375 of the *E. coli* EF-Tu protein by valine or threonine results in an EF-Tu molecule with an increased resistance to elfamycin (F. Duisterwinkel et al., FEBS Letters 13 (1981) 89-93, F. Duisterwinkel et al., EMBO J. 3 (1984) 113-120).

Several techniques can be employed to introduce similar mutations into the DNA encoding the EF-Tu protein of *S. ramocissimus*. In a preferred embodiment the pMa-c vector system and *E. coli* host strains WK6 and WK6mutS are employed (P. Stanssens et al. Nucl. Acid. Res. 17, (1989) 4441-4454), in combination with gapped-duplex mutagenesis (W. Kramer et al. Nucl. Acid. Res. 12 (1984) 9441-9456). Specifically synthetic oligonucleotide probes were designed and used to mutagenize the alanine at position 378 in EF-Tu from *S. ramocissimus* to valine (A378V), threonine (A378T), proline (A378P), or phenylalanine (A378F). Other possibilities for mutation are yet other amino acid residues at position 378, or e.g. mutation of glutamic acid at position 360 into phenylalanine.

To obtain the modified protein the mutated gene can be expressed in any suitable host; examples are given of expression in *E. coli* and in *S. ramocissimus*.

The sensitivity of SrEF-Tu mutants A378V and A378T to elfamycin was tested by *in vitro* studies. Both parent and mutant *S. ramocissimus* EF-Tu, after transformation of the respective cloned genes, were expressed in an *E. coli* strain encoding an elfamycin resistant EF-Tu. Cell-free extracts of these transformants were subsequently tested for elfamycin sensitivity of the translation apparatus, using a variation on the procedure described by C. Glöckner and H. Wolf (cited above). It was found that the SrEF-Tu mutants A378V and A378T had a residual activity of 50% at an elfamycin concentration of 160 mg/l. The parent SrEF-Tu reached 50% residual activity already at 1.6 mg/l. Therefore, elfamycin resistant EF-Tu proteins are considered to be proteins with a residual activity of 50% at an elfamycin concentration of at least 2 mg/ml, when tested in the above assay.

All mutant EF-Tu proteins obtained were tested through direct binding studies visualized by a change in migration in non-denaturing PAGE upon elfamycin binding. Each mutant EF-Tu (A378V, A378T, A378P, and A378F) proved to be unable to bind the elfamycin in this assay.

The mutated genes are introduced into the mocimycin producing host. In a preferred embodiment this is *S. ramocissimus*. Preferably, the mutated gene is integrated into the chromosome. To that purpose an integration vector can be used, having homology with the tuf gene locus. Integration is then preferably performed at this locus, whereby the parent gene is replaced by the mutated gene. The mutated gene can also be inserted into the chromosome at other loci of choice, preferably loci where the expression level of the encoded protein is high. For high level expression of the protein, plasmid location is also possible and can be advantageous. In the latter two cases (insertion of the tufR at another locus than the tuf gene and plasmid encoded tufR) it is essential that the parent gene is inactivated by mutation, e.g. deletion of the complete gene or a part thereof or of the regulating sequences.

The *S. ramocissimus* strain in which the chromosomal Srtuf1 gene was replaced by tufR was found to have its resistance level towards elfamycin increased more than 5-fold in the vegetative mycelial growth phase. In addition resistance towards the effects of exogenous elfamycins on sporulation and germination of spores was equally increased. An elfamycin resistant Streptomyces is defined as a strain characterized in that its spores germinate and grow in YMG medium containing yeast extract 4 g/l, malt extract 10 g/l and glucose 4 g/l, in the presence of at least 0.2 g elfamycin/l preferably 0.2-1.0 g elfamycin/l.

In contrast to *Escherichia coli* no adverse effects of the EF-Tu mutation on the growth rate of the elfamycin resistant *Streptomyces ramocissimus* was observed.

As demonstrated the EF-TuR proteins of this invention will give rise to strains with an increased resistance against elfamycins. Elfamycin production will be increased or at least if measures are taken to increase the elfamycin production the strains containing the modified proteins will be capable of increased elfamycin production.

The following examples will illustrate the invention, without in any way limiting its scope.

In the examples, unless otherwise specified, all procedures for making and manipulating recombinant DNA using *E. coli* as a host were carried out by standardized procedures described by T. Maniatis et al. (1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.)

Example 1Isolation and characterization of Elongation Factor Tu from *S. ramocissimus* (SrEF-Tu)

5 *S. ramocissimus* CBS 190.69 was cultured in liquid S-medium (M. Okanishi et al., J. Gen. Microbiol. 80 (1974) 389-400) for 72 hrs at 30 °C. Mycelium was harvested by centrifugation and resuspended in icecold standard buffer (10 mM Tris/HCl pH 7.8, 60 mM NH₄Cl, 10 mM Mg-acetate, 1 mM DTT, 0.1% PMSF). The suspension was sonicated at 0 °C with 10 bursts of 45 seconds, allowing 15 seconds in between for cooling. The sonicated suspension was centrifuged at 30000 g for 15 minutes. The ribosomes still present in the
 10 resulting S-30 extract were pelleted by centrifugation for 3 hr at 100000 g. The supernatant of this centrifugation was regarded as the S-100 fraction of *S. ramocissimus* mycelium.

SrEF-Tu was purified by affinity chromatography on GDP-AH-Sepharose (G. Jacobson and J. Rosenbusch, cited above). This procedure yielded a single component protein preparation as judged by SDS-PAGE. The protein migrated with an apparent molecular weight of 50 kD, whereas *E. coli* EF-Tu migrates at
 15 45 kD. The purified protein was identified as *S. ramocissimus* EF-Tu (SrEF-Tu) by analysis of the protein by GDP exchange experiments (H. Weissbach et al., cited above) and by its ability to promote EF-Tu dependent poly(U) directed synthesis of polyphenylalanine using *E. coli* ribosomes.

Both elfamycin binding studies (G. Chinali et al., cited above) and the inhibition by added elfamycin of the in vitro poly(U) translation system directed by SrEF-Tu indicated that SrEF-Tu is elfamycin sensitive
 20 (see Example 5, and also C. Glöckner and H. Wolf, cited above).

The purified SrEF-Tu was used to raise polyclonal antibodies in rabbits according to standard techniques.

Example 2

25

Identification, isolation and characterization of the *S. ramocissimus* tuf genes

The procedure used to isolate *S. ramocissimus* CBS 190.69 chromosomal DNA was essentially that described by D. Hopwood et al. (cited above). Southern blotting experiments of this DNA, digested with
 30 restriction enzymes, showed that an *E. coli* tufA probe (HpaI/NruI fragment, T. Yokota et al., cited above) hybridized strongly with a BglII fragment of approximately 3.0 kb and less strongly but very specifically to a 3.0 kb BamHI fragment and a 4.2 kb PstI fragment.

For cloning of the strongly hybridizing DNA fragment, *S. ramocissimus* chromosomal DNA was digested to completion with BglII and ligated with BamHI digested plasmids pUC8 (J. Vieira and J. Messing, Gene 19
 35 (1982) 259-268) and pUC18 (C. Yanisch-Perron et al. Gene 33 (1985) 103-119), respectively. The host for transformation was *E. coli* strain JM101 (J. Messing, Recombinant DNA Technical Bulletin 2 (1979) 43-48).

A sib-selection procedure was applied to screen pools of transformants for the presence of *S. ramocissimus* tuf sequences. With this procedure the initial selection by Southern hybridization is applied to
 40 plasmids isolated from a pool of transformants. A positive pool is successively reduced in size and in each step the total plasmid population of the pool is screened by Southern hybridization. Finally plasmids isolated from single transformants are analyzed.

In the sib-selection procedure to isolate the *S. ramocissimus* tuf gene, plasmid DNA from 11 pools of 50-70 transformants each was isolated and electrophoresed on agarose gels. Southern hybridization of these DNA preparations with the *E. coli* tufA probe revealed one positive pool. Successive reduction of the
 45 pool size resulted in one positive recombinant pUC18 plasmid containing a BglII insert of 2.8 kb. This plasmid was designated pUSrT1 (Figure 2a).

The complete 2.8 kb fragment was sequenced on both strands using the chain termination method of Sanger et al. (cited above), and M13mp18 or M13mp19 phages (C. Yanisch-Perron, cited above) as vector. Analysis of the sequence revealed an open reading frame of 1191 bp, encoding a protein of 397 amino
 50 acids, including the N-terminal methionine (Figure 1; SEQ ID 1). The protein sequence derived from this open reading frame showed a 74% homology with *E. coli* EF-Tu. The gene cloned on pUSrT1 was considered to be the *S. ramocissimus* EF-Tu encoding gene Sruf1.

Similarly, using the Sruf1 gene as a hybridization probe, the 3.0 kb BamHI fragment harboring the Sruf2 gene and the 4.2 kb PstI fragment harboring the Sruf3 gene were cloned and the nucleotide
 55 sequences of the coding regions determined. The coding sequences of Sruf2 and Sruf3 and the derived amino acid sequences of the encoded products SrEF-Tu2 and SrEF-Tu3 are listed as SEQ ID 2 and SEQ ID 3, respectively. SrEF-Tu2 has 71% and SrEF-Tu3 64% of its amino acid residues identical to *E. coli* EF-Tu.

Example 3Heterologous expression of the Srtuf1 gene in E. coli

For an independent identification and characterization of the *S. ramocissimus* EF-Tu protein, the cloned Srtuf1 gene was expressed in *E. coli* JM101. Expression was obtained by placing the Srtuf gene downstream of the inducible lac promoter on the *E. coli* plasmids pUC18 as follows:

The NruI/XbaI fragment of pUSrT1 containing the Srtuf gene was isolated, ligated with SmaI/XbaI digested pUC18, and transformed to *E. coli* JM101 yielding plasmid pUSrT1-1 (Figure 2b).

Growth of *E. coli* JM101 transformed with pUSrT1-1 and induction of the lac promoter was achieved by culturing the transformants for 16 hrs at 37 °C in LB-medium supplemented with 100 µg/ml ampicillin and 0.5 mM IPTG.

Total protein of these cells was analyzed using SDS-PAGE. This revealed the presence of a new protein species in the transformed *E. coli*. This protein comigrated with purified SrEF-Tu, and reacted strongly with SrEF-Tu antibodies (see Example 1) in Western blotting experiments.

This experiment thus identified the gene present on pUSrT1-1, as the Srtuf1 gene encoding a protein called SrEF-Tu1.

For purification of SrEF-Tu1 an S-100 fraction of *E. coli* JM101/pUSrT1-1 cells was prepared (Example 1), stabilized by the addition of GDP to 25 µM and passed through a GDP-AH Sepharose column. Under these conditions the *E. coli* EF-Tu is bound to the column, whereas the SrEF-Tu1 protein passes through. The GDP-stabilized eluate was then applied to a Dyematrix RED-A column (Amicon). After elimination of unbound protein, the SrEF-Tu1 was eluted at approximately 0.45 M NaCl by applying a linear salt gradient from 0 to 1.5 M NaCl.

Example 4Site directed mutagenesis of Srtuf1

For site directed mutagenesis of the Srtuf1 gene the pMa-c vector system and *E. coli* host strains WK6 and WK6mutS (P. Stanssens et al., cited above) were employed in combination with the gapped-duplex mutagenesis protocol (W. Kramer et al., cited above).

pUSrT1 was digested with EcoRI and HindIII and the Srtuf gene containing fragment was ligated into EcoRI and HindIII digested pMa6 and pMc6 yielding plasmids pMaSrT1 and pMcSrT1, respectively (Figure 3). pMa6 and pMc6 are derivatives of plasmids pMa5-8 and pMc5-8 (P. Stanssens et al., cited above), lacking the PstI site within the β -lactamase gene.

The mutagenesis and mutant selection procedure was performed using plasmids pMaSrT1 and pMcSrT1, essentially as described by P. Stanssens et al. (cited above). In short, single-stranded DNA was prepared from plasmid pMcSrT1 by infection of pMcSrT1 containing *E. coli* JM101 cells with phage M13KO7. For formation of the gapped duplex, single-stranded pMcSrT1 was combined with the larger MluI/XbaI fragment of pMaSrT1 and either synthetic oligonucleotide 1 (SEQ ID 4), or synthetic oligonucleotide 2 (SEQ ID 5) to mutate position 378 (alanine) of the SrEF-Tu1 protein to valine and threonine. The mutant proteins were designated SrEF-Tu A378V and A378T, respectively. After gap-filling and ligation using DNA polymerase I (large fragment) and T4-DNA ligase, the samples were transformed to *E. coli* WK6mutS, while selecting for ampicillin resistance. Next, plasmid DNA was isolated from pooled WK6mutS transformants and introduced into *E. coli* strain WK6. Individual ampicillin resistant WK6 transformants were subsequently infected with M13KO7 as described above in order to obtain plasmid DNA in single-stranded form. Nucleotide sequence analysis (see Example 2) was used to identify clones containing the desired mutation, and to ascertain that no secondary mutations had been introduced within the gap during the mutagenesis procedure.

Plasmids containing the respective desired mutations were recovered and designated pMaSrT1V and pMaSrT1T (Figure 3).

Similarly, the mutations A378P (proline) and A378F (phenylalanine) were introduced using mutagenic oligonucleotides 3 (SEQ ID 6) and 4 (SEQ ID 7), respectively. Plasmids obtained by these experiment were designated pMaSrT1P and pMaSrT1F.

Example 5Properties of SrEF-Tu mutants A378V and A378T in an in vitro peptide synthesis assay

In order to obtain expression of the mutant *Srtuf* genes, the larger *MluI/XbaI* fragment of pUSrT1-1 was ligated with the smaller *MluI/XbaI* fragment of both pMaSrT1V and pMaSrT1T, yielding plasmids pUSrT1V-1 and pUSrT1T-1, respectively (Figure 2).

Plasmids pUSrT1-1, pUSrT1V-1 and pUSrT1T-1 were transformed to *E. coli* PM1455 (*tufA*, *tufB*::Mu, *rpoB*, *recA56*; P. van der Meide et al., Eur. J. Biochem. 130 (1983) 409-417); this strain has only one active *tuf* gene, which encodes an elfamycin resistant EF-Tu. The respective *E. coli* PM1455 transformants were grown as described in Example 3, and an S-30 extract was prepared essentially as described in Example 1. One ml of the extract was applied on a 10 ml Sephadex G-25 column (2 g of Sephadex G-25) of 15-20 cm length (10 ml pipet). The column was eluted with standard buffer (Example 1) and fractions of 5 drops (500-700 μ l) were collected. The first 4 fractions having absorbance at 260 nm were pooled. This crude pooled fraction was used for promoting *in vitro* poly(U) directed poly(phe) synthesis as follows.

At 0°C an incubation mixture was prepared consisting of 40 mM Tris-acetate pH 7.6, 10 mM Mg-acetate, 60 mM NH₄Cl, 5 mM β -mercaptoethanol, 1 mM ATP, 0.025 mM GTP, 2.5 mM phosphoenolpyruvate, 0.25 μ g/ml pyruvate kinase, 0.8 mg/ml tRNA, 0.1 mg/ml poly(U), 95 μ M phenylalanine, and 3 μ Ci/ml ³H-phenylalanine (57 Ci/mmol). To 0.6 ml of this incubation mixture 0.12 ml crude extract was added. Subsequently 50 μ l samples were incubated at 37°C. Incubation mixtures were processed as follows: 150 μ l 100 mM NaOH was added and incubation was prolonged for 5 minutes at 37°C. Next 800 μ l 5% trichloroacetic acid (TCA) was added and the samples were stored at 0°C for 5 minutes. The precipitate was filtered over GFC filters (Whatman), washed three times with 5% TCA and once with 96% ethanol. Then the filters were dried for 30 minutes at 80°C, 2 ml xylene scintillation fluid was added to each filter. Incorporation of ³H-phenylalanine was analyzed in a liquid scintillation counter.

In different experiments either the elfamycin concentrations or the incubation times were varied as indicated in Figure 4. The result of the first experiment is displayed in Figure 4a. Increasing amounts of mocimycin were added to incubation mixtures in parallel poly(phe) synthesis experiments using the S-30 extract mentioned above. Reaction times were kept constant at 10 minutes. Both SrEF-Tu mutants A378V and A378T displayed a residual activity of 50% in the *in vitro* poly(phe) synthesis in the presence of 160 mg/l mocimycin, whereas a residual activity of 50% for the parent SrEF-Tu from *S. ramocissimus* CBS 190.69 was already observed at 1.6 mg/l mocimycin (Figure 4a).

In the second experiment, the synthesis of poly(phe) over a period of 40 minutes at a mocimycin concentration of 16 mg/l was studied. It was found that during this incubation, ³H-phenylalanine incorporation directed by both SrEF-Tu mutants A378V and A378T, proceeds with an efficiency of 80% if compared to the parallel incubation without mocimycin. In the control experiment, S-30 extracts containing parent SrEF-Tu in the presence of 16 mg/l mocimycin performed with a maximum efficiency of 20% of the mocimycin free reaction (Figure 4b).

Example 6

Visualization of elfamycin binding of SrEF-Tu mutants A378V, A378T, A378P, and A378F by non-denaturing PAGE

For direct visualization of the elfamycin binding capacity, the mutant proteins were expressed in *E. coli* JM101 essentially as described in Example 3. Subsequently, a GDP stabilized S-30, S-100, or purified SrEF-Tu sample was prepared, and incubated with 25 μ M aurodox for 15 minutes at 37°C. These samples, and control samples without aurodox were then subjected to non-denaturing 10% polyacrylamide gels and electrophoresed. Detection of the SrEF-Tu species was performed by the Western blotting technique using SrEF-Tu antibodies (Example 1). Whereas wild-type *S. ramocissimus* EF-Tu appears to bind the elfamycin as indicated by the increased migration of the ternary complex into the gel (Figure 5), the migration of the mutant SrEF-Tu proteins A378V, A378T, A378P, and A378F was unaffected by preincubation with the elfamycin. This experiment thus established that elfamycin resistance is most likely the effect of a reduced binding of the elfamycin to the mutant SrEF-Tu proteins.

Example 7

Construction of the gene replacement vectors pMTST1V Δ S and pMTST1T Δ S

In order to obtain plasmids pMTST1V Δ S and pMTST1T Δ S capable of replacing the parent chromosomal *Srtuf* gene, several intermediate constructs were prepared.

pUt18:

Plasmid pIJ702 (E. Katz et al., J. Gen. Microbiol. 129 (1983) 2703-2714) was digested with BclI, the 1.05 kb fragment containing the thiostrepton resistance gene was purified and subsequently ligated into BamHI digested pUC18. Transformation of *E. coli* JM101 yielded the desired plasmid pUt18 (Figure 6a).

pStT1V-1 and pStT1T-1:

pUt18 was digested with SmaI and HindIII and the 1.1 kb fragment containing the thiostrepton resistance gene was purified.

pUSrT1V-1 and pUSrT1T-1 were digested with EcoRI and HindIII and the 1.9 kb fragment containing the mutated Srtuf gene was purified.

Both purified fragments were combined with pSP70 (Promega) digested with PvuII and EcoRI, ligated and transformed to *E. coli* JM101. Plasmids containing all three of the above elements were identified and named pStT1V-1 and pStT1T-1, respectively (Figure 6b).

pStT1VΔS and pStT1TΔS:

The upstream region and 5' coding region of the mutant Srtuf gene was deleted from plasmids pStT1V-1 and pStT1T-1 by digestion with EcoRI and SmaI, followed by treatment with DNA polymerase I (large fragment) to convert the sticky EcoRI ends to blunt ends, ligation, and transformation to *E. coli* JM101. The desired constructs were obtained and named pStT1VΔS and pStT1TΔS (Figure 7a).

pMTST1VΔS and pMTST1TΔS:

The larger fragments resulting from PstI/PvuII digestion of pStT1VΔS and pMT660 (A. Birch and J. Cullum, J. Gen. Microbiol. 131 (1985) 1299-1303), respectively, were ligated and transformed to *E. coli* JM101. Thus plasmids pMTST1VΔS was obtained. Similarly, starting from pStT1TΔS, plasmid pMTST1TΔS was constructed (Figure 7b).

Example 8**Replacement of the parent *S. ramocissimus* *tuf1* gene by a mutated *tuf1* gene encoding an elfamycin resistant EF-Tu protein**

For replacement of the parent *S. ramocissimus* EF-Tu encoding gene by the mutant elfamycin resistant EF-Tu variant genes A378V and A378T, fresh spores of *S. ramocissimus* CBS 190.69 were prepared using sporulation medium of the following composition: NaNO_3 0.3 g/l, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ 0.2 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.005 g/l, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g/l, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g/l, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.005 g/l, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.04 g/l, L-Methionine 0.1 g/l, L-Leucine 0.1 g/l, L-Tyrosine 0.5 g/l, glucose 10 g/l, and agar 20 g/l. Starting from a culture in S-medium (Example 1), 0.5 ml was spread on sporulation plates and incubated at 30°C for 5 days.

Spores were isolated essentially as described by D. Hopwood et al. (cited above), and used to inoculate YMG medium (yeast extract 4 g/l, malt extract 10 g/l, glucose 4 g/l) containing 0.5% glycine. Protoplasts were obtained by lysozyme treatment of this culture, transformed as described by D. Hopwood et al. (cited above) with plasmid pMTST1VΔS and pMTST1TΔS. Subsequently the transformed protoplasts were spread on regeneration medium, and incubated at 30°C. Regeneration medium was prepared by mixing equal volumes of sporulation medium and stabilizer medium. Stabilizer medium consisted of NaNO_3 3 g/l, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ 0.085 g/l, K_2SO_4 0.25 g/l, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g/l, trace element solution 0.1 ml, Tris 3.03 g/l, NaCl 2.92 g/l, sucrose 103 g/l, glucose 10 g/l, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 5 g/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.5 g/l, and agar 20 g/l, adjusted to pH 7.2 with 4N HCl. Trace element solution had the following composition: $\text{Fe}(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ 0.25 g/l, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 g/l, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.04 g/l, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.015 g/l, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.015 g/l, H_3BO_3 0.005 g/l, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 0.0055 g/l, KI 0.01 g/l, adjusted to pH 3.0 with 4N HCl. After 24 hrs the regeneration plates were overlaid with 3 ml soft agar containing 20 µg/ml thiostrepton (D. Hopwood et al., cited above) and incubated at 30°C for 5 days.

Thiostrepton resistant colonies were streaked on sporulation medium containing 2 µg/ml thiostrepton, and individual colonies were cultured at 30°C in YMG medium containing 2 µg/ml thiostrepton. Subsequently plasmid DNA was isolated from each culture and analyzed by restriction enzyme mapping to

confirm the identity and integrity of the transformed plasmids pMTST1VΔS and pMTST1TΔS.

To obtain integration of plasmid pMTST1VΔS in the chromosome of *S. ramocissimus* CBS 190.69, preferably by homologous recombination of the plasmid located mutant *SrtufR* sequences with the parent *Srtuf1* locus (Figure 8a), use was made of the temperature sensitive pMT660 replicon. Selected transformants were passed through several (at least 3) cycles of culturing in liquid medium (YMG) and sporulation at 37 °C in the presence of 2 µg/ml thiostrepton, in order to remove freely replicating plasmid from the cells, but to select for chromosomal integration of the plasmid. Spores obtained by this procedure were diluted, plated, and incubated at 37 °C; individual colonies were picked, grown at 37 °C in YMG containing 2 µg/ml thiostrepton, and checked for the absence of plasmid DNA. Next, total DNA was isolated from plasmid free colonies, digested with *Bgl*III and analyzed by Southern blotting. Integration was observed through disappearance of the chromosomal 2.8 kb band and appearance of both a 1.2 and a 9.2 kb band (Figure 8b).

Strains having one plasmid copy integrated into the chromosomal *Srtuf1* locus, were grown in YMG without thiostrepton and plated on non-selective sporulation medium. Spores were isolated, diluted, and plated on non-selective sporulation medium. Subsequent replica plating of single colonies to sporulation medium containing 2 µg/ml thiostrepton identified thiostrepton sensitive strains which had lost the plasmid sequences by intramolecular homologous recombination of the chromosome (the reverse process of plasmid integration). Selection of thiostrepton sensitive strains for elfamycin resistance both in liquid YMG medium containing 0.5 g/l mocimycin and on solid sporulation medium containing 0.1 g/l mocimycin yielded strain *S. ramocissimus* R1V having a restored chromosomal *Srtuf* locus which is identical to parent *S. ramocissimus* CBS 190.69, except for the A378V mutation (Figure 8c).

Similarly, plasmid pMTST1TΔS can be used to obtain *S. ramocissimus* strain R1T having the parent *Srtuf* locus, except for the mutation A378T.

Example 9

Elfamycin resistance properties of *S. ramocissimus* strain R1V

Spores, mycelium, and protoplasts of strain *S. ramocissimus* R1V were examined with respect to the minimal inhibitory concentration of mocimycin on their growth properties.

Spores of strain *S. ramocissimus* R1V and the control *S. ramocissimus* CBS 190.69 were inoculated at $5 \cdot 10^7$ spores/ml in parallel shake flasks containing 25 ml YMG medium and mocimycin at concentrations ranging from 0 to 1 g/l. Incubation was for 5 days at 30 °C. Table 1 illustrates the results of this experiment. For the control strain 0.15 g/l mocimycin inhibited germination (and/or growth) of the spores, whereas spores of *S. ramocissimus* strain R1V still germinated and grew at mocimycin concentrations up to 0.75 g/l.

Similar results were obtained on solid medium (HI-agar, Difco) containing 0 to 1 g/l mocimycin; spores from *S. ramocissimus* R1V and *S. ramocissimus* CBS 190.69 were diluted such that approximately 200 colony forming units were applied to each agar plate. Incubation of the plates was at 30 °C for 5 days. For the control strain *S. ramocissimus* CBS 190.69 no colonies appeared above 0.1 g/l mocimycin; on the contrary, spores of *S. ramocissimus* strain R1V quantitatively were able to germinate and form colonies at least up to 0.75 g/l.

Essentially identical results were obtained when for each of the strains spores were substituted by mycelium, pregrown in YMG medium without mocimycin. Plating 1 ml of a 16 hrs culture it was found that strain *S. ramocissimus* R1V and the control *S. ramocissimus* CBS 190.69 had minimal inhibitory concentrations of 0.75 and 0.15 g/l, respectively.

Another test was carried out using protoplasts of strains *S. ramocissimus* CBS 190.69 and *S. ramocissimus* R1V, prepared as described in Example 7. As protoplasts lack the cell wall, which forms a protective barrier between the intracellular compartment and the external medium, they exhibit a considerably higher sensitivity towards elfamycin than do spores or mycelium. Thus protoplasts were plated on regeneration medium (Example 7) containing 0 to 0.1 g/l mocimycin. Under these conditions protoplasts of *S. ramocissimus* CBS 190.69 were able to regenerate only at mocimycin concentrations below 0.02 g/l; regeneration of *S. ramocissimus* R1V protoplasts in the presence of up to 0.1 g/l mocimycin occurred with the same efficiency as without mocimycin (Table 2).

Table 1

5 Germination and growth of S. ramocissimus spores on HI-agar containing varying amounts of mocimycin.

	Mocimycin concentration (g/l)						
	0	0.10	0.20	0.40	0.60	0.75	1.0
10 15 <u>S. ramocissimus</u> CBS 169.90	+++	+	-	-	-	-	-
20 <u>S. ramocissimus</u> R1V	+++	+++	+++	++	++	+	-

+++ = good growth; ++ = slow growth; + = very slow growth;
- = no growth

Table 2

30 Regeneration efficiency of S. ramocissimus protoplasts on regeneration medium at different mocimycin concentrations.

	Mocimycin concentration (g/l)					
	0.00	0.01	0.02	0.04	0.07	0.10
40 <u>S. ramocissimus</u> CBS 190.69	100%	40%	0%	0%	0%	0%
45 <u>S. ramocissimus</u> R1V	100%	100%	100%	100%	90%	80%

50 Example 10

Analysis of EF-TuR isolated from S. ramocissimus strain R1V

55 To establish that S. ramocissimus strain R1V actually expresses the mutated Srtuf1 gene as the main EF-Tu species, an S-100 extract was prepared from a culture of S. ramocissimus strain R1V essentially as described in Example 1. Subsequently this extract was subjected to the direct elfamycin binding assay outlined in Example 6. The results of this experiment, shown in Figure 9, prove that the major EF-Tu

species of *S. ramocissimus* strain R1V contrary to that of strain CBS 190.69 is unable to bind the elfamycin aurodox under the conditions employed.

5

SEQUENCE LISTING

SEQ ID NO : 1

SEQUENCE TYPE : Nucleotide with corresponding protein

10

SEQUENCE LENGTH : 1194 base pairs

STRANDEDNESS : double-stranded

TOPOLOGY : Linear

MOLECULE TYPE : Genomic DNA

15

ORIGINAL SOURCE : Streptomyces ramocissimus

STRAIN : CBS 190.69

FEATURES : from 1 to 1191 bp: coding sequence

from 1 to 396 aa : translation elongation factor Tuf1 protein

20

PROPERTY : Streptomyces ramocissimus tuf1 gene, encoding translation elongation factor Tuf1

25

GTG	GCG	AAG	GCG	AAG	TTC	GAG	CGG	ACT	AAG	CCG	CAC	GTC	AAC	ATC	GGC	48
Ala	Lys	Ala	Lys	Phe	Glu	Arg	Thr	Lys	Pro	His	Val	Asn	Ile	Gly		
1				5				10						15		

30

ACC	ATC	GGT	CAC	ATC	GAC	CAC	GGT	AAG	ACG	ACC	CTC	ACG	GCC	GCC	ATT	96
Thr	Ile	Gly	His	Ile	Asp	His	Gly	Lys	Thr	Leu	Thr	Ala	Ala	Ile		
			20					25						30		

35

ACC	AAG	GTG	CTG	CAC	GAC	GCG	TAC	CCG	GAC	CTG	AAC	GAG	GCC	ACC	CCG	144
Thr	Lys	Val	Leu	His	Asp	Ala	Tyr	Pro	Asp	Leu	Asn	Glu	Ala	Thr	Pro	
			35					40						45		

40

TTC	GAC	AAC	ATC	GAC	AAG	GCT	CCT	GAG	GAG	CGT	CAG	CGC	GGT	ATC	ACC	192
Phe	Asp	Asn	Ile	Asp	Lys	Ala	Pro	Glu	Glu	Arg	Gln	Arg	Gly	Ile	Thr	
			50					55						60		

ATC	TCC	ATC	GCG	CAC	GTC	GAG	TAC	CAG	ACC	GAG	GCG	CGT	CAC	TAC	GCC	240
Ile	Ser	Ile	Ala	His	Val	Glu	Tyr	Gln	Thr	Glu	Ala	Arg	His	Tyr	Ala	
			65					70						75		

45

CAC	GTC	GAC	TGC	CCG	GGT	CAC	GCG	GAC	TAC	ATC	AAG	AAC	ATG	ATC	ACG	288
His	Val	Asp	Cys	Pro	Gly	His	Ala	Asp	Tyr	Ile	Lys	Asn	Met	Ile	Thr	
			80					85						90		

50

GGT	GCG	GCG	CAG	ATG	GAC	GGC	GCC	ATC	CTC	GTG	GTC	GCC	GCC	ACC	GAC	336
Gly	Ala	Ala	Gln	Met	Asp	Gly	Ala	Ile	Leu	Val	Val	Ala	Ala	Thr	Asp	
			100					105						110		

GGC	CCG	ATG	CCG	CAG	ACC	AAG	GAG	CAC	GTG	CTC	CTG	GCC	CGC	CAG	GTC	384
Gly	Pro	Met	Pro	Gln	Thr	Lys	Glu	His	Val	Leu	Leu	Ala	Arg	Gln	Val	
			115					120						125		

55

	GGC GTT CCG TAC ATC GTG GTC GCC CTG AAC AAG GGC GAC ATG GTG GAC	432
	Gly Val Pro Tyr Ile Val Val Ala Leu Asn Lys Ala Asp Met Val Asp	
	130 135 140	
5	GAC GAG GAG ATC ATG GAG CTC GTT GAG CTC GAG GTC CGT GAG CTC CTC	480
	Asp Glu Glu Ile Met Glu Leu Val Glu Leu Glu Val Arg Glu Leu Leu	
	145 150 155	
10	TCC GAG TAC GAG TTC CCG GGC GAC GAC CTG CCG GTC GTC CGC GTC TCC	528
	Ser Glu Tyr Glu Phe Pro Gly Asp Asp Leu Pro Val Val Arg Val Ser	
	160 165 170 175	
	GCG CTG AAG GCG CTG GAG GGC GAC GCT CAG TGG ACG CAG TCC GTC CTC	576
	Ala Leu Lys Ala Leu Glu Gly Asp Ala Gln Trp Thr Gln Ser Val Leu	
15	180 185 190	
	GAC CTG ATG AAG GCC GTC GAC GAG TCC ATC CCG GAG CCG GAG CGC GAC	624
	Asp Leu Met Lys Ala Val Asp Glu Ser Ile Pro Glu Pro Glu Arg Asp	
	195 200 205	
20	GTC GAC AAG CCG TTC CTC ATG CCG ATC GAG GAC GTC TTC ACG ATC ACC	672
	Val Asp Lys Pro Phe Leu Met Pro Ile Glu Asp Val Phe Thr Ile Thr	
	210 215 220	
	GGT CGC GGC ACG GTC GTC ACC GGC CGT ATC GAG CGT GGT GTC CTG AAG	720
25	Gly Arg Gly Thr Val Val Thr Gly Arg Ile Glu Arg Gly Val Leu Lys	
	225 230 235	
	GTC AAC GAG ACC GTC GAC ATC ATC GGC ATC AAG ACC GAG AAG ACC ACC	768
	Val Asn Glu Thr Val Asp Ile Ile Gly Ile Lys Thr Glu Lys Thr Thr	
	240 245 250 255	
30	ACC ACG GTC ACC GGC ATC GAG ATG TTC CGC AAG CTG CTC GAC GAG GGC	816
	Thr Thr Val Thr Gly Ile Glu Met Phe Arg Lys Leu Leu Asp Glu Gly	
	260 265 270	
	CAG GCC GGT GAG AAC GTC GGT CTG CTG CTC CGC GGC ATC AAG CGC GAG	864
35	Gln Ala Gly Glu Asn Val Gly Leu Leu Leu Arg Gly Ile Lys Arg Glu	
	275 280 285	
	GAC GTC GAG CGC GGC CAG GTC ATC ATC AAG CCG GGC TCG GTC ACC CCG	912
	Asp Val Glu Arg Gly Gln Val Ile Ile Lys Pro Gly Ser Val Thr Pro	
40	290 295 300	
	CAC ACC GAG TTC GAG GCG CAG GCC TAC ATC CTC TCC AAG GAC GAG GGT	960
	His Thr Glu Phe Glu Ala Gln Ala Tyr Ile Leu Ser Lys Asp Glu Gly	
	305 310 315	
45	GGC CGC CAC ACG CCG TTC TTC AAC AAC TAC CGC CCG CAG TTC TAC TTC	1008
	Gly Arg His Thr Pro Phe Phe Asn Asn Tyr Arg Pro Gln Phe Tyr Phe	
	320 325 330 335	
	CGT ACC ACG GAC GTG ACC GGC GTT GTG CAC CTC CCC GAG GGC ACC GAG	1056
50	Arg Thr Thr Asp Val Thr Gly Val Val His Leu Pro Glu Gly Thr Glu	
	340 345 350	
55		

	ATG GTC ATG CCG GGC GAC AAC ACC GAG ATG CGC GTC GAG CTG ATC CAG	1104
	Met Val Met Pro Gly Asp Asn Thr Glu Met Arg Val Glu Leu Ile Gln	
5	355 360 365	
	CCC GTC GCC ATG GAG GAG GGC CTG AAG TTC GCC ATC CGT GAG GGT GGC	1152
	Pro Val Ala Met Glu Glu Gly Leu Lys Phe Ala Ile Arg Glu Gly Gly	
	370 375 380	
10	CGG ACC GTC GGC GCC GGC CAG GTC ACC AAG ATC GTC AAG TAA	1194
	Arg Thr Val Gly Ala Gly Gln Val Thr Lys Ile Val Lys	
	385 390 395	
15		
20		
25		
30		
35		
40		
45		
50		
55		

SEQ ID NO : 2

SEQUENCE TYPE : Nucleotide with corresponding protein

SEQUENCE LENGTH : 1194 base pairs

5 STRANDEDNESS : double-stranded

TOPOLOGY : Linear

MOLECULE TYPE : Genomic DNA

10 ORIGINAL SOURCE : Streptomyces ramocissimus

STRAIN : CBS 190.69

FEATURES : from 1 to 1191 bp: coding sequence

from 1 to 396 aa : translation elongation factor Tu2 protein

15 PROPERTY : Streptomyces ramocissimus tuf2 gene, encoding translation
elongation factor Tu2

20

GTG GCG AAG GCG AAG TTC CAG CGG ACC AAA CCG CAC GTC AAC ATC GGC	48
Ala Lys Ala Lys Phe Gln Arg Thr Lys Pro His Val Asn Ile Gly	
1 5 10 15	

25

ACC ATC GGC CAC ATC GAC CAC GGC AAG ACG ACA CTC ACC GCG GCG ATC	96
Thr Ile Gly His Ile Asp His Gly Lys Thr Thr Leu Thr Ala Ala Ile	
20 25 30	

30

ACG AAG GTG CTG CAC GAC CGG TTC CCC GAC CTC AAC CCG TTC ACC CCG	144
Thr Lys Val Leu His Asp Arg Phe Pro Asp Leu Asn Pro Phe Thr Pro	
35 40 45	

35

TTC GAC CAG ATC GAC AAG GCG CCC GAG GAA CGG CAG CGC GGC ATC ACC	192
Phe Asp Gln Ile Asp Lys Ala Pro Glu Glu Arg Gln Arg Gly Ile Thr	
50 55 60	

40

ATC TCG ATC GCC CAC GTC GAG TAC CAG ACC GAG GCG CGG CAC TAC GCG	240
Ile Ser Ile Ala His Val Glu Tyr Gln Thr Glu Ala Arg His Tyr Ala	
65 70 75	

45

CAC GTC GAC TGC CCC GGA CAC GCC GAC TAC ATC AAG AAC ATG ATC ACC	288
His Val Asp Cys Pro Gly His Ala Asp Tyr Ile Lys Asn Met Ile Thr	
80 85 90 95	

GGC GCG GCC CAG ATG GAC GGC GCG ATC CTG GTC GTC GCG GCC ACG GAC	336
Gly Ala Ala Gln Met Asp Gly Ala Ile Leu Val Val Ala Ala Thr Asp	
100 105 110	

50

GGG CCG ATG CCC CAG ACC AAG GAA CAT GTG CTG CTG GCA CGG CAG GTG	384
Gly Pro Met Pro Gln Thr Lys Glu His Val Leu Leu Ala Arg Gln Val	
115 120 125	

55

5	GGC GTG CCC TAC ATC GTC GTC GCG CTG AAC AAG ACC GAC ATG GTC GAC	432
	Gly Val Pro Tyr Ile Val Val Ala Leu Asn Lys Thr Asp Met Val Asp	
	130 135 140	
	GAC GAG GAG ATC CTC GAA CTC GTG GAG TTG GAG GTG CGC GAG CTG CTC	480
	Asp Glu Glu Ile Leu Glu Leu Val Glu Leu Glu Val Arg Glu Leu Leu	
10	ACC GAG TAC GAG TTC CCC GGC GAC GAC GTC CCG GTC GTC AAG GTG TCG	528
	Thr Glu Tyr Glu Phe Pro Gly Asp Asp Val Pro Val Val Lys Val Ser	
	160 165 170 175	
	GCG CTC AGG GCC CTG GAG GGC GAC CCC CGG TGG ACC CGG TCG GTA CTC	576
	Ala Leu Arg Ala Leu Glu Gly Asp Pro Arg Trp Thr Arg Ser Val Leu	
15	180 185 190	
	GAA CTC CTC GAC GCC GTC GAC GAG TTC GTG CCC GAG CCG GTG CGG GAC	624
	Glu Leu Leu Asp Ala Val Asp Glu Phe Val Pro Glu Pro Val Arg Asp	
	195 200 205	
	GTC GAC CGG CCG TTC CTG ATG CCG ATC GAG GAC GTC TTC ACC ATC ACC	672
20	Val Asp Arg Pro Phe Leu Met Pro Ile Glu Asp Val Phe Thr Ile Thr	
	210 215 220	
	GGA CGC GGC ACG GTC GTC ACC GGC CGG ATA GAG CGC GGC ACG CTG AAC	720
	Gly Arg Gly Thr Val Val Thr Gly Arg Ile Glu Arg Gly Thr Leu Asn	
	225 230 235	
25	GTG AAC ACC GAG GTG GAG ATC ATC GGC ATC CAC GAA CAG AGG ACC CGG	768
	Val Asn Thr Glu Val Glu Ile Ile Gly Ile His Glu Gln Arg Thr Arg	
	240 245 250 255	
	ACC ACG GTC ACC GGC ATC GAG ATG TTC CCG AAG CTC CTC GAC GAG GGC	816
	Thr Thr Val Thr Gly Ile Glu Met Phe Arg Lys Leu Leu Asp Glu Gly	
30	260 265 270	
	CGG GCC GGC GAG AAC GTC GGA CTG CTG CTG CGC GGA GTG AAG CGG GAG	864
	Arg Ala Gly Glu Asn Val Gly Leu Leu Leu Arg Gly Val Lys Arg Glu	
	275 280 285	
	CAG GTC GAG CGC GGT CAG GTC GTC ATC AGG CCC GGA TCG GTC ACC CCG	912
35	Gln Val Glu Arg Gly Gln Val Val Ile Arg Pro Gly Ser Val Thr Pro	
	290 295 300	
	CAC ACG CAG TTC GAG GCG CAG GCG TAC ATC CTG TCC AAG GAC GAG GGC	960
	His Thr Gln Phe Glu Ala Gln Ala Tyr Ile Leu Ser Lys Asp Glu Gly	
	305 310 315	
40	GGC CGG CAC ACG CCG TTC TTC GAG AAC TAC CGT CCG CAG TTC TAC TTC	1008
	Gly Arg His Thr Pro Phe Phe Glu Asn Tyr Arg Pro Gln Phe Tyr Phe	
	320 325 330 335	
	CGC ACC ACC GAC GTC ACG GGC GTG GTG ACG CTG CCG AAG GGC ACC GAG	1056
	Arg Thr Thr Asp Val Thr Gly Val Val Thr Leu Pro Lys Gly Thr Glu	
45	340 345 350	
	55	

5	ATG GTG ATG CCG GGC GAC AAC ACC GCC ATG CAC GTC CAG CTG ATC CAG	1104
	Met Val Met Pro Gly Asp Asn Thr Ala Met His Val Gln Leu Ile Gln	
	355 360 365	
10	CCG ATC GCC ATG GAG GAG GGG CTG AAG TTC GCC ATC CGC GAG GGC GGG	1152
	Pro Ile Ala Met Glu Glu Gly Leu Lys Phe Ala Ile Arg Glu Gly Gly	
	370 375 380	
15	CGC ACG GTC GGC GCC GGC CAG GTC ACG CGG ATC GTG AAG TAG	1194
	Arg Thr Val Gly Ala Gly Gln Val Thr Arg Ile Val Lys	
	385 390 395	
20		
25		
30		
35		
40		
45		
50		
55		

SEQ ID NO : 3

SEQUENCE TYPE : Nucleotide with corresponding protein

5 SEQUENCE LENGTH : 1170 base pairs

STRANDEDNESS : double-stranded

TOPOLOGY : Linear

MOLECULE TYPE : Genomic DNA

10 ORIGINAL SOURCE : Streptomyces ramocissimus

STRAIN : CBS 190.69

FEATURES : from 1 to 1167 bp: coding sequence

15 from 1 to 388 aa : translation elongation factor Tu3 protein

PROPERTY : Streptomyces ramocissimus tuf3 gene, encoding translation
elongation factor Tu3

20

ATG TCC AAG ACG GCA TAC GTG CGC ACC AAA CCG CAT CTG AAC ATC GGC	48
Ser Lys Thr Ala Tyr Val Arg Thr Lys Pro His Leu Asn Ile Gly	
1 5 10 15	

25

ACG ATG GGT CAT GTC GAC CAC GGC AAG ACC ACG TTG ACC GCC GCC ATC	96
Thr Met Gly His Val Asp His Gly Lys Thr Leu Thr Ala Ala Ile	
20 25 30	

30

ACC AAG GTC CTC GCC GAG CGT GGC TCC GGG ACG TTC GTC CCG TTC GAC	144
Thr Lys Val Leu Ala Glu Arg Gly Ser Gly Thr Phe Val Pro Phe Asp	
35 40 45	

35

CGC ATC GAC CGG GCC CCG GAG GAG GCC GCG CGC GGC ATC ACC ATC AAC	192
Arg Ile Asp Arg Ala Pro Glu Glu Ala Ala Arg Gly Ile Thr Ile Asn	
50 55 60	

40

ATC GCG CAC GTC GAG TAC GAG ACC GAC ACC CGG CAC TAC GCG CAC GTC	240
Ile Ala His Val Glu Tyr Glu Thr Asp Thr Arg His Tyr Ala His Val	
65 70 75	

45

GAC ATG CCG GGC CAC GCC GAC TAC GTC AAG AAC ATG GTC ACC GGC GCC	288
Asp Met Pro Gly His Ala Asp Tyr Val Lys Asn Met Val Thr Gly Ala	
80 85 90 95	

50

GCG CAG CTC GAC GGG GCG ATC CTC GTC GTC TCC GCG CTC GAC GGG ATC	336
Ala Gln Leu Asp Gly Ala Ile Leu Val Val Ser Ala Leu Asp Gly Ile	
100 105 110	

55

ATG CCG CAG ACC GCC GAA CAC GTC CTG CTC GCC CGG CAG GTG GGC GTC	384
Met Pro Gln Thr Ala Glu His Val Leu Leu Ala Arg Gln Val Gly Val	
115 120 125	

	GAC CAC ATC GTC GTC GCC CTC AAC AAG GCC GAC GCG GGC GAC GAG GAG	432
	Asp His Ile Val Val Ala Leu Asn Lys Ala Asp Ala Gly Asp Glu Glu	
	130 135 140	
5	CTC ACC GAC CTC GTC GAG CTG GAG GTC CGC GAT CTG CTC TCC GAG CAC	480
	Leu Thr Asp Leu Val Glu Leu Glu Val Arg Asp Leu Leu Ser Glu His	
	145 150 155	
10	GGC TAC GGC GGC GAC GGT GCC CCC GTC GTA CGG GTC TCG GGG CTG AAG	528
	Gly Tyr Gly Gly Asp Gly Ala Pro Val Val Arg Val Ser Gly Leu Lys	
	160 165 170 175	
15	GCG CTG GAG GGC GAC CCC AAG TGG ACG GCG TCC ATC GAG GCG CTG CTC	576
	Ala Leu Glu Gly Asp Pro Lys Trp Thr Ala Ser Ile Glu Ala Leu Leu	
	180 185 190	
	GAC GCG GTG GAC ACC TAC GTG CCG ATG CCG GAG CCG TAT GTG GAC GCG	624
	Asp Ala Val Asp Thr Tyr Val Pro Met Pro Glu Arg Tyr Val Asp Ala	
	195 200 205	
20	CCG TTC CTG CTG CCC GTG GAG AAC GTG CTC ACC ATC ACC GGT CCG GGC	672
	Pro Phe Leu Leu Pro Val Glu Asn Val Leu Thr Ile Thr Gly Arg Gly	
	210 215 220	
25	ACC GTG GTC ACC GGA GCC GTC GAG CGG GGC ACC GTG CCG GTG GGC AAC	720
	Thr Val Val Thr Gly Ala Val Glu Arg Gly Thr Val Arg Val Gly Asn	
	225 230 235	
	CGG GTC GAA GTG CTC GGC GCC GGG CTG GAG ACC GTG GTC ACC GGC CTG	768
	Arg Val Glu Val Leu Glu Ala Gly Leu Glu Thr Val Val Thr Gly Leu	
	240 245 250 255	
30	GAG ACG TTC GGC AAG CCC ATG GAC GAG GCG CAG GCC GGG GAC AAC GTG	816
	Glu Thr Phe Gly Lys Pro Met Asp Glu Ala Gln Ala Gly Asp Asn Val	
	260 265 270	
35	GCG CTG TTG CTG CGT GGG GTT CCG CGG GAC GCC GTA CCG CGT GGG CAT	864
	Ala Leu Leu Leu Arg Gly Val Pro Arg Asp Ala Val Arg Arg Gly His	
	275 280 285	
40	GTG GTC GCG GCG CCG GGG AGC GTG GTG CCC CGG AGT CGA TTC TCC GCG	912
	Val Val Ala Ala Pro Gly Ser Val Val Pro Arg Ser Arg Phe Ser Ala	
	290 295 300	
	CAG GTG TAT GTG CTC TCG GCC CGC GAG GGC GGT CGT ACG ACT CCT GTC	960
	Gln Val Tyr Val Leu Ser Ala Arg Glu Gly Gly Arg Thr Thr Pro Val	
	305 310 315	
45	ACC AGC GGG TAT CGG CCG CAG TTC TAC ATC CGT ACG GCG GAT GTG GTG	1008
	Thr Ser Gly Tyr Arg Pro Gln Phe Tyr Ile Arg Thr Ala Asp Val Val	
	320 325 330 335	
50	GGG GAC GTC GAC CTG GGG GAG GTG GGG GTC GCT CGG CCT GGG GAG ACG	1056
	Gly Asp Val Asp Leu Gly Glu Val Gly Val Ala Arg Pro Gly Glu Thr	
	340 345 350	
55		

5 GTT TCG ATG ATC GTC GAG TTG GGC CGG GAG GTT CCG CTG GAG CCC GGG 1104
 Val Ser Met Ile Val Glu Leu Gly Arg Glu Val Pro Leu Glu Pro Gly
 355 360 365
 10 TTG GGG TTC GCC ATT CGT GAG GGC GGC AGG ACC GTG GGG GCG GGG ACC 1152
 Leu Gly Phe Ala Ile Arg Glu Gly Gly Arg Thr Val Gly Ala Gly Thr
 370 375 380
 15 GTT ACG GCC CTT GTG TGA 1170
 20 Val Thr Ala Leu Val
 385

15 SEQ ID NO : 4
 SEQUENCE TYPE : Nucleotide
 SEQUENCE LENGTH : 33 nucleotides
 20 STRANDEDNESS : single-stranded
 TOPOLOGY : Linear
 MOLECULE TYPE : Synthetic DNA
 25 PROPERTY : Oligonucleotide changing the codon for alanine 378 of the
 S.ramocissimus Srtuf1 gene to valine.

30 GCCACCCTCA CGGATGACGA ACTTCAGGCC CTC 33

35 SEQ ID NO : 5
 SEQUENCE TYPE : Nucleotide
 SEQUENCE LENGTH : 33 nucleotides
 40 STRANDEDNESS : single-stranded
 TOPOLOGY : Linear
 MOLECULE TYPE : Synthetic DNA
 PROPERTY : Oligonucleotide changing the codon for alanine 378 of the
 45 S.ramocissimus Srtuf1 gene to threonine.

50 GCCACCCTCA CGGATGGTGA ACTTCAGGCC CTC 33

55

SEQ ID NO : 6

SEQUENCE TYPE : Nucleotide

5 SEQUENCE LENGTH : 33 nucleotides

STRANDEDNESS : single-stranded

TOPOLOGY : Linear

MOLECULE TYPE : Synthetic DNA

10 PROPERTY : Oligonucleotide changing the codon for alanine 378 of the
S.ramocissimus Srtufl gene to proline.

15 GCCACCTCA CGGATCGGA ACTTCAGGCC CTC 33

20 SEQ ID NO : 7

SEQUENCE TYPE : Nucleotide

SEQUENCE LENGTH : 33 nucleotides

25 STRANDEDNESS : single-stranded

TOPOLOGY : Linear

MOLECULE TYPE : Synthetic DNA

30 PROPERTY : Oligonucleotide changing the codon for alanine 378 of the
S.ramocissimus Srtufl gene to phenylalanine.

35 GCCACCTCA CGGATGAAGAA CTCAGGCC CTC 33

Claims

- 40
1. Protein EF-TuR, characterized in that it is obtainable from an elfamycin producing actinomycete and made elfamycin resistant.
 2. Protein EF-TuR according to Claim 1, characterized in that the elfamycin is mocimycin (kirromycin).
 - 45 3. Protein EF-TuR according to Claim 1 or 2, characterized in that, when tested in a cell-free system for poly (U)-directed polyphenylalanine synthesis, it has a residual activity of 50% in a medium containing at least 2 mg per liter mocimycin, preferably at least 160 mg per liter mocimycin.
 - 50 4. Protein EF-TuR according to any of Claims 1-3, characterized in that the actinomycete is a streptomycete, preferably Streptomyces ramocissimus, more preferably Streptomyces ramocissimus CBS 190.69.
 - 55 5. Protein EF-TuR according to any of Claims 1-4, consisting of an amino acid sequence corresponding by at least 80% to that depicted in Figure 1 of the accompanying drawings.
 6. Protein EF-TuR according to Claim 5, characterized in that the alanine at position 378 or at the position corresponding thereto in an homologous protein, is replaced by either valine, threonine, proline, or

phenylalanine.

7. A DNA sequence *tufR* encoding protein EF-TuR according to any one of Claims 1-6.
- 5 8. A DNA sequence *tufR* according to Claim 7, as depicted in Figure 1 of the drawings, characterized in that the codon encoding the alanine at position 378, or at the position corresponding thereto in an homologous gene, is replaced by one encoding valine, threonine, proline, or phenylalanine.
9. A vector containing a DNA sequence according to Claim 7 or 8, preferably the vector is plasmid.
- 10 10. A plasmid vector characterized in that it is pMaSrT1V, pMaSrT1T, pMaSrT1P, pMaSrT1F, pUSrT1V-1, pUSrT1T-1, pUSrT1P-1, pUSrT1F-1, pStT1V-1, pStT1T-1, pStT1VΔS, pStT1TΔS, pMTST1VΔS, or pMTST1TΔS, as depicted in Figures 2, 3, 6, and 7 of the accompanying drawings.
- 15 11. An elfamycin producing actinomycete, comprising a DNA sequence *tufR* according to Claim 7 or 8.
12. An elfamycin producing actinomycete according to Claim 11, characterized in that the elfamycin is mocimycin.
- 20 13. An elfamycin producing actinomycete according to Claim 11 or 12, wherein said DNA sequence *tufR* is integrated in the chromosome, replacing the DNA sequence *tuf*.
14. An elfamycin producing actinomycete according to any of Claims 11-13, characterized in that it is a streptomycete.
- 25 15. A mocimycin producing streptomycete characterized in that its spores germinate and grow in YMG medium containing yeast extract 4 g/l, malt extract 10 g/l and glucose 4 g/l, in the presence of at least 0.2 g/l preferably 0.2-1.0 g/l mocimycin.
- 30 16. A streptomycete according to Claim 15, characterized in that it belongs to the species Streptomyces ramocissimus.
17. Streptomyces ramocissimus strain R1V derived from Streptomyces ramocissimus CBS 190.69, expressing the gene encoding the mutant protein SrEF-Tu A378V.
- 35 18. Process for obtaining an elfamycin producing actinomycete expressing an elfamycin resistant EF-TuR according to any of Claims 1-6, comprising the following steps:
 1. cloning of the gene *tuf* from an elfamycin producing actinomycete,
 2. applying site-directed mutagenesis on said gene *tuf*, thereby altering the gene *tuf* encoding an elfamycin sensitive EF-Tu into the gene *tufR* encoding an elfamycin resistant EF-TuR,
 - 40 3. constructing a vector containing the gene *tufR* or a part thereof,
 4. transforming an elfamycin producing actinomycete by introducing said vector into it,
 5. selecting said transformant for integration of said vector into the chromosomal *tuf* locus, by its elfamycin resistant phenotype.
- 45 19. A process for the preparation of an elfamycin comprising the fermentation of an actinomycete capable of producing an elfamycin and which actinomycete is resistant to an elfamycin concentration of at least 0.2 g/l, preferably 0.2-1.0 g/l.
- 50 20. A process according to Claim 19 characterized in that the elfamycin is mocimycin.
21. A process according to Claim 19 or 20 characterized in that the actinomycete is a streptomycete, preferably Streptomyces ramocissimus.

55

Figure 1

```

      30                               60
GTGGCGAAGGCGAAGTTCGAGCGGACTAAGCCGCACGTCAACATCGGCACCATCGGTAC
  A K A K F E R T K P H V N I G T I G H
  1

      90                               120
ATCGACCACGGTAAGACGACCCTCACGGCCGCCATTACCAAGGTGCTGCACGACGCGTAC
  I D H G K T T L T A A I T K V L H D A Y
  20

     150                               180
CCGGACCTGAACGAGGCCACCCCGTTTCGACAACATCGACAAGGCTCCTGAGGAGCGTCAG
  P D L N E A T P F D N I D K A P E E R Q
  40

     210                               240
CGCGGTATCACCATCTCCATCGCGCAGTCCGAGTACCAGACCGAGGCGCGTCACTACGCC
  R G I T I S I A H V E Y Q T E A R H Y A
  60

     270                               300
CACGTGCACTGCCCGGGTCACGCGGACTACATCAAGAACATGATCACGGGTGCGGCGCAG
  H V D C P G H A D Y I K N M I T G A A Q
  80

     330                               360
ATGGACGGCGCCATCCTCGTGGTCGCCGCCACCGACGGCCCGATGCCGCAGACCAAGGAG
  M D G A I L V V A A T D G P M P Q T K E
  100

     390                               420
CACGTGCTCCTGGCCCGCCAGGTCCGGCTTCCGTACATCGTGGTCGCCCTGAACAAGGCC
  H V L L A R Q V G V P Y I V V A L N K A
  120

     450                               480
GACATGGTGGACGACGAGGAGATCATGGAGCTCGTTGAGCTCGAGGTCCGTGAGCTCCTC
  D M V D D E E I M E L V E L E V R E L L
  140

     510                               540
TCCGAGTACGAGTTCCCGGGCGACGACCTGCCGGTCGTCCGCGTCTCCGCGCTGAAGGCG
  S E Y E F P G D D L P V V R V S A L K A
  160

     570                               600
CTGGAGGGCGACGCTCAGTGGACGAGTCCGTCCTCGACCTGATGAAGGCCGTCGACGAG
  L E G D A Q W T Q S V L D L M K A V D E
  180

     630                               660
TCCATCCCGGAGCCGGAGCGCGACGTGACAAGCCGTTCTCATGCCGATCGAGGACGTC
  S I P E P E R D V D K P F L M P I E D V
  200

```


690 720
 TTCACGATCACC GGTCGCGGCACGGTCGTCACCGGCCGTATCGAGCGTGGTGTCTGAAG
 F T I T G R G T V V T G R I E R G V L K
 220

750 780
 GTCAACGAGACCGTCGACATCATCGGCATCAAGACCGAGAAGACCACCACCGTCACC
 V N E T V D I I G I K T E K T T T T V T
 240

810 840
 GGCATCGAGATGTTCCGCAAGCTGCTCGACGAGGGCCAGGCCGGTGAGAACGTGCGTCTG
 G I E M F R K L L D E G Q A G E N V G L
 260

870 900
 CTGCTCCGCGGCATCAAGCGCGAGGACGTGAGCGCGGCCAGGTCATCATCAAGCCGGGC
 L L R G I K R E D V E R G Q V I I K P G
 280

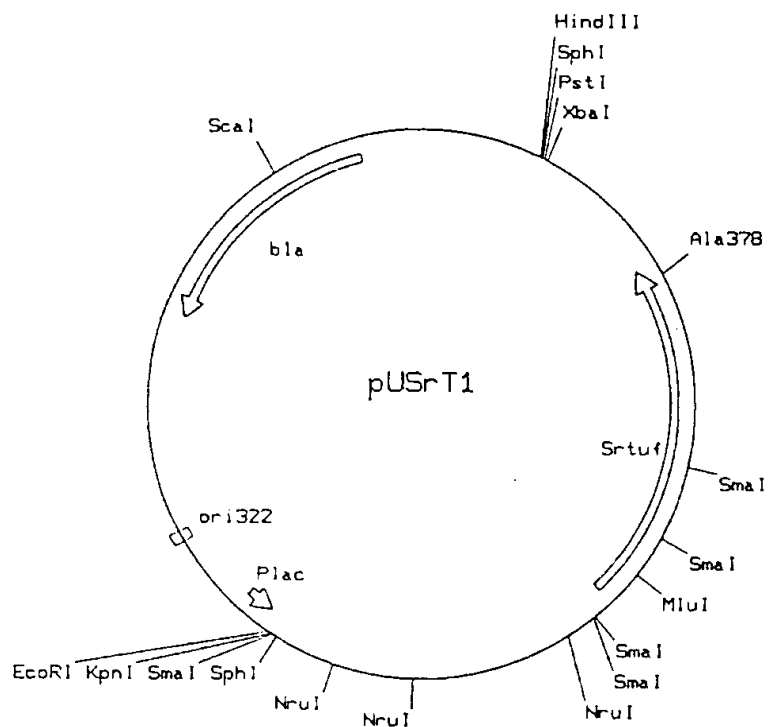
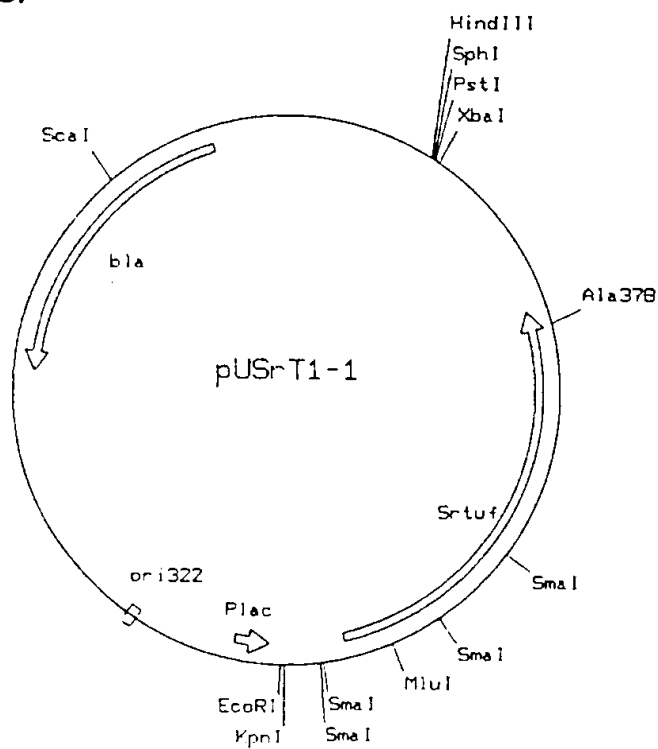
930 960
 TCGGTCACCCCGCACACCGAGTTCGAGGGCGCAGGCCTACATCCTCTCCAAGGACGAGGGT
 S V T P H T E F E A Q A Y I L S K D E G
 300

990 1020
 GGCCGCCACACGCCGTTCTTCAACAACCTACCGCCCGCAGTTCTACTTCCGTACCACGGAC
 G R H T P F F N N Y R P Q F Y F R T T D
 320

1050 1080
 GTGACCGGCGTGTGTGCACCTCCCCGAGGGCACCGAGATGGTCATGCCGGGCGACAACACC
 V T G V V H L P E G T E M V M P G D N T
 340

1110 1140
 GAGATGCGCGTCGAGCTGATCCAGCCCGTCGCCATGGAGGAGGGCCTGAAGTTCGCCATC
 E M R V E L I Q P V A M E E G L K F A I
 360 378

1170
 CGTGAGGGTGGCCGACCGTCGCGCGCGGCCAGGTCACCAAGATCGTCAAGTAA
 R E G G R T V G A G Q V T K I V K *
 380

Figure 2 a.**b.**

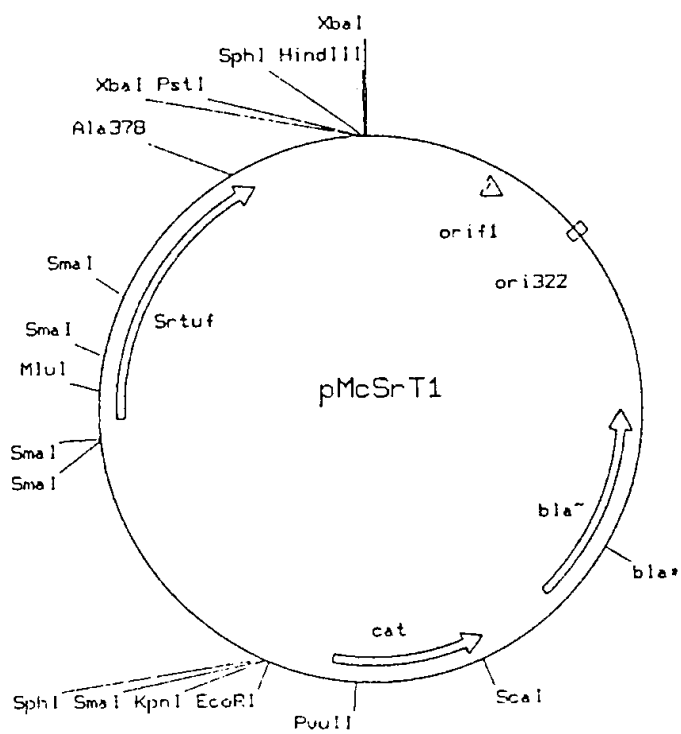


Figure 4 a.

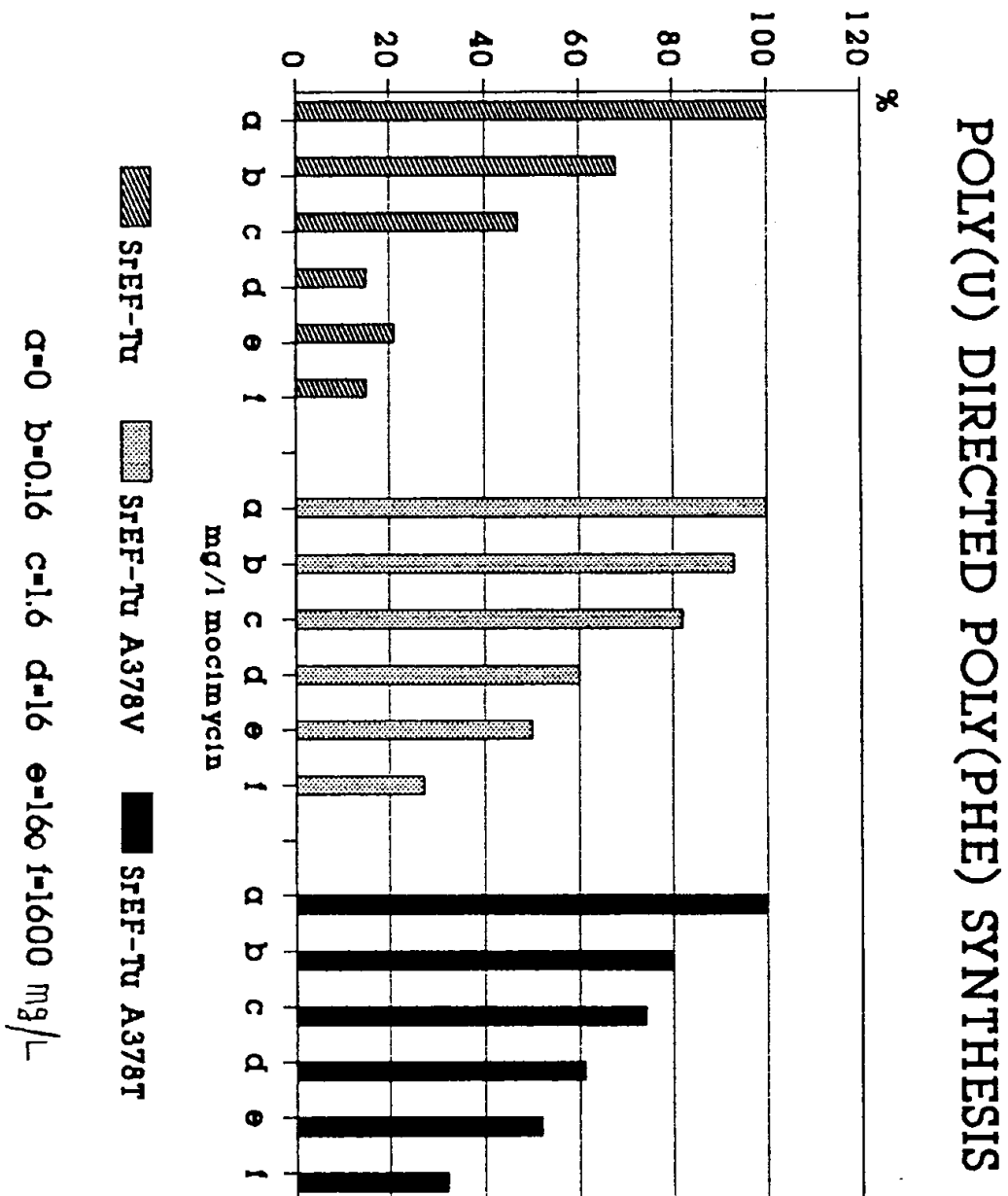
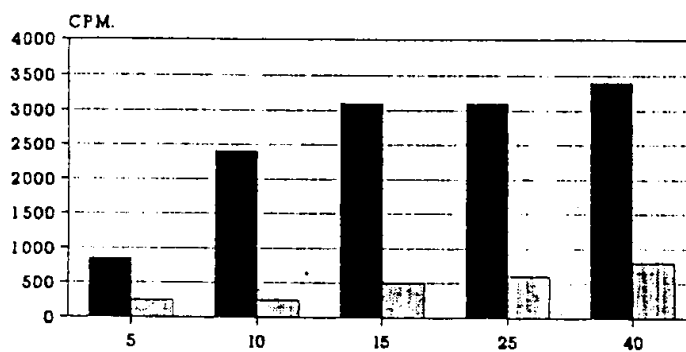
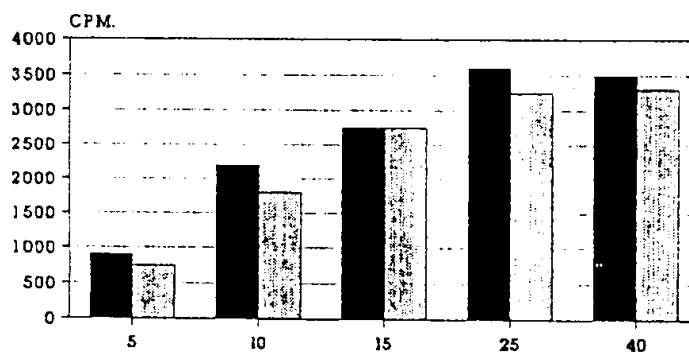


Figure 4 b.

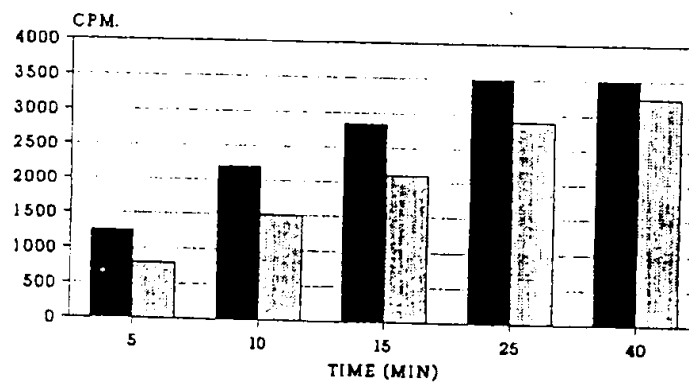
a. POLY(U) DIRECTED POLY(PHE) SYNTHESIS
SrEF-Tu



b. SrEF-Tu A378V



c. SrEF-Tu A378T



■ - MOCIMYCIN ▨ + MOCIMYCIN 16 mg/l

Figure 5

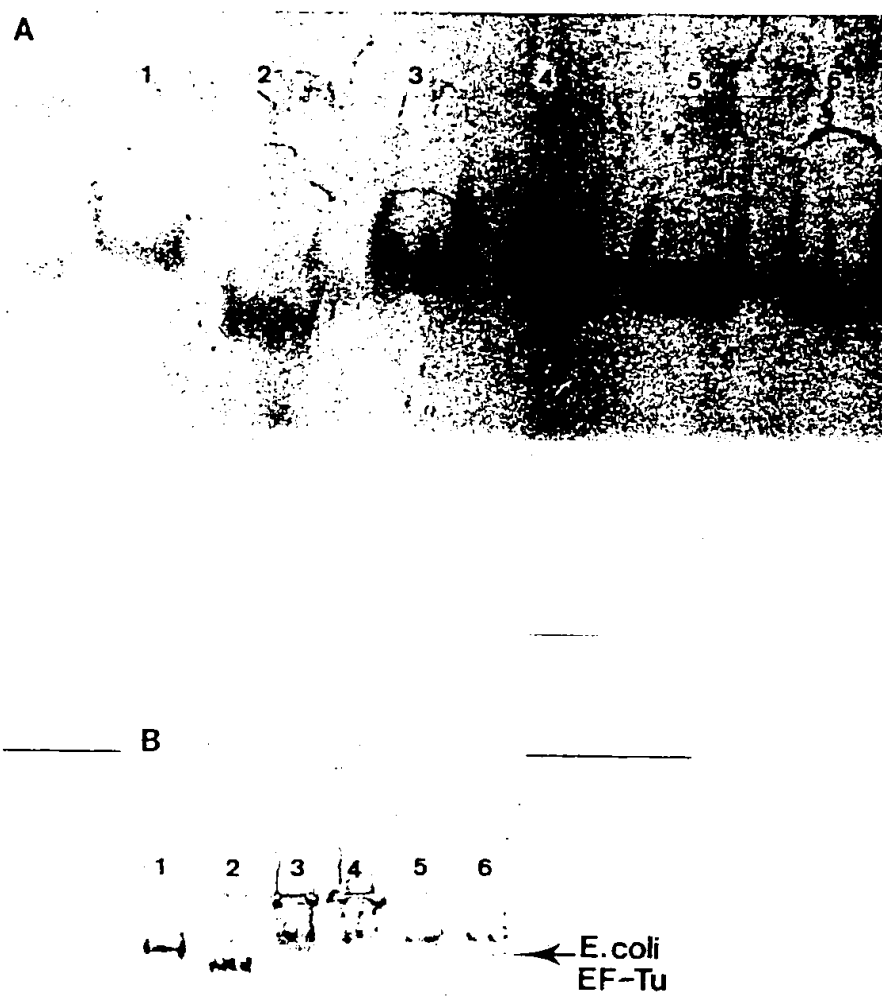


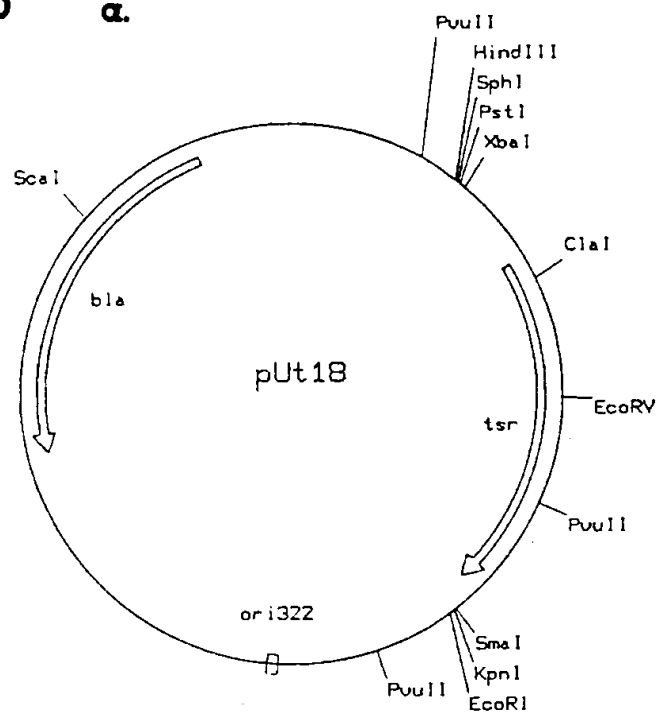
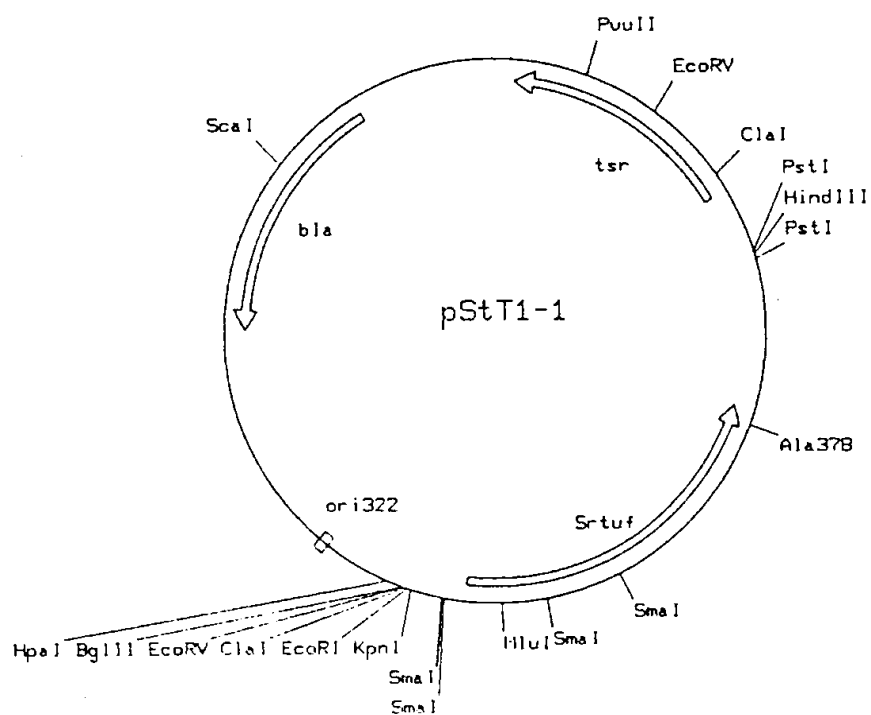
Figure 6**a.****b.**

Figure 7 a.

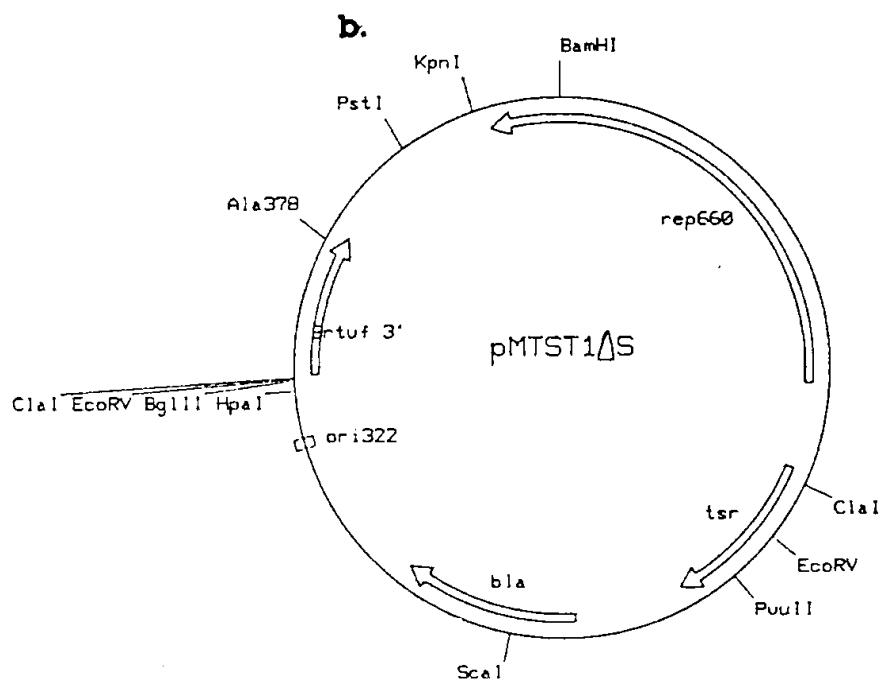
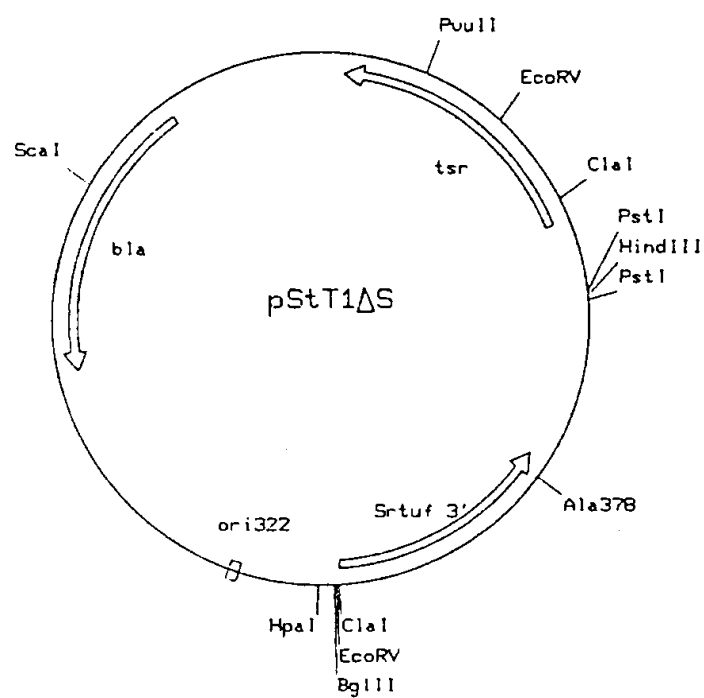
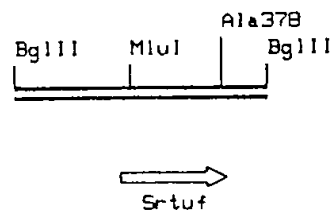
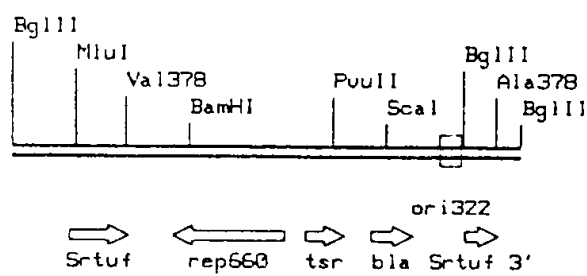


Figure 8 **a.**



b.



c.

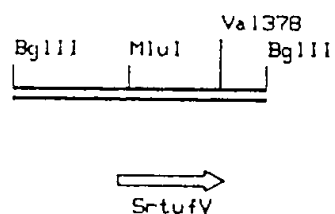


Figure 9





European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 20 1702

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	HEREDITY vol. 61, no. 2, 1988, EDINBURGH pages 291 - 292; WOUDT B. ET AL: 'Analysis of TUF-genes from Streptomyces ramocissimus ' * abstract *	1-21	C12N15/31 C12P21/02 C12N1/21 C12R1/465 C12P19/02
Y	BIOCHIMIE vol. 69, no. 10, October 1987, PARIS VIJGENBOOM E. ET AL: 'Transfer of plasmid-borne TUF mutations to the chromosome as a genetic tool for studying the functioning of EF-TuA and EF-TuB in the E.coli cell " ' * the whole document *	1-21	
D,Y	EMBO JOURNAL vol. 3, no. 1, 1984, EYNSHAM, OXFORD GB pages 113 - 120; DUISTERWIJN F.J. ET AL: 'Specific alterations of the Ef-Tu polypeptide chain considered in the light of its three-dimensionnal structure ' * the whole document *	1-21	
D,X	FEMS MICROBIOLOGY LETTERS vol. 25, 1984, AMSTERDAM pages 121 - 124; GLOCKNER C. ET AL: 'Mechanism of natural resistance to kirromycin type antibiotics in Actinomycetes ' * the whole document , especially page 123-124 *	15-16, 19-21	
D,A		1-14, 17-18	
X	US-A-3 927 211 (GIST-BROCADES N.V.) * column 8, line 21 - line 62; claim 3 *	15-16, 19-21	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 09 OCTOBER 1991	Examiner LE CORNEC N.D.R.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons # : member of the same patent family, corresponding document			